BIOPHARMACEUTICAL ASPECTS RELEVANT TO PULMONARY TARGETING OF INHALED GLUCOCORTICOIDS: APPLICATION TO LIPOSOMES AND DRY POWDERS

By

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DEDICATED TO MY PARENTS ELENA URIBE TREJO AND JOAQUIN SUAREZ VILLAGRAN AND MY SISTERS CARMEN, ROSALBA, RAQUEL, LOURDES, ELENA AND MY BROTHERS JOAQUIN, ARTURO, EDUARDO, ROBERTO AND ROGELIO.

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Abstract of Dissertation Presented to the Graduate School
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BIOPHARMACEUTICAL ASPECTS OF INHALED GLUCOCORTICOIDS:
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Little information is available regarding the biopharmaceutical factors that govern pulmonary targeting. Pharmacokinetics/pharmacodynamic simulations have shown that pulmonary targeting can be improved by optimization of dose and drug release rate. Liposomes were used as a model dosage form to show the relevance of dose and release rate on pulmonary targeting in *in vivo* situations. Liposomes were composed of two phospholipids and triamcinolone acetonide phosphate (TAP-lip). TAP-lip and the drug in solution were administered intratrachealy (IT) into anesthetized rats. Pulmonary targeting was determined by simultaneously monitoring receptor occupancy in lung and liver. The IT administration of the TAP-lip formulation resulted in higher pulmonary targeting and longer mean pulmonary effect times (MET) than that after the IT administration of the drug in solution. Thus, liposomes may represent a valuable approach to optimize the delivery of glucocorticoids via topical administration. The pharmacokinetics and

pharmacodynamics of triamcinolone acetonide (TA) after IT and intravenous (IV) administration of TAP-lip, and after IT instillation of the drug in solution, were also investigated. Plasma and lung TA concentrations were determined by HPLC/RIA. No changes in the pharmacodynamics (EC_{50} and E_{max}), were observed, indicating that pharmacokinetics is the driving force to enhance pulmonary targeting. The intratracheal administration of TAP in different release rate preparations resulted in mean effect times (MET) and pulmonary targeting which were higher for the slowest release preparation. The administration of escalating doses of TAP in 800 nm liposomes showed a bell shaped curve, suggesting that there is an optimum dose for which maximum pulmonary targeting can be achieved. Differences in pulmonary targeting between triamcinolone acetonide (TA) and fluticasone propionate (FP) dry-powders were assessed after IT administration by the *ex vivo* receptor assay. No significant differences in pulmonary targeting were observed between these two drugs. Higher pulmonary targeting and longer MET values were obtained after the IT administration of the liposomal formulation than those after the IT administration of TA-dry powder. These studies clearly demonstrate the relevance of dose and release rate for the optimization of pulmonary selectivity as well as the suitability of the *ex vivo* animal model to assess pulmonary targeting.

CHAPTER 1
INTRODUCTION

Asthma is an inflammatory disease of the airways in which many cells play a role including eosinophils and mast cells. Asthma is perhaps the only treatable condition whose severity is on the rise. This problem requires the development of drugs which are more effective and safer for the treatment of the disease.

Inhaled glucocorticoids have become first-line therapy for the treatment of moderate to severe asthma as a results of glucocorticoid action at the cellular level to attenuate the underlying disease process. These drugs are administered directly into the respiratory airways using different devices such as metered dose inhaler (with and without spacer devices), nebulizer and dry powder inhaler.

Inhalation therapy makes possible the administration of small doses of the drug directly into the target site, a rapid onset of action as well as a low systemic exposure, minimizing the risk of systemic side effects.

Several studies have shown that pulmonary selectivity is possible if a higher local to systemic drug ratio is achieved. In attempts to increase this ratio drugs with high systemic clearance and low oral bioavailability have been introduced. Thus, the latest generations of inhaled glucocorticoids have oral bioavailability values lower than <1% (fluticasone propionate) and clearance values closer to the hepatic blood flow (1.2 L/min). Despite the advantages of inhaled glucocorticoids over oral therapy, there are still

concerns regarding side effects, especially suppression of growth in children and osteoporosis, since inhaled glucocorticoids are likely to be administered over long periods of time.

Recent computer simulation studies [1] have shown that pulmonary targeting can be modulated if drug release rate and dose are optimized. These theoretical studies demonstrated that there is an optimal dose and release rate for which maximum pulmonary targeting can be achieved. It was important to demonstrate using *in vivo* experiments whether these two factors are relevant in pulmonary selectivity. To show the importance of these two factors, liposomes were used as a model dosage form due to their flexibility in release rate characteristics, and their compatibility with lung surfactant components. Furthermore, since inhaled glucocorticoids differ in their dissolution rate *in vitro* it was also important to explore differences in release rate among these drugs (triamcinolone acetonide and fluticasone propionate) based on the fact that pulmonary targeting is affected by release rate.

CHAPTER 2
BACKGROUND

2.1 Asthma

2.1.1 Definition

Asthma is considered an inflammatory disease, often described as chronic desquamating eosinophilic bronchitis [2]. Asthma is a common disease of the airways which affects 5% of the adult and 10-15% of the child population [3, 4]. Asthma has become more prevalent during the last decades, and the severity and mortality have increased [5]. Asthma is generally characterized by a combination of cough, dyspnea, chest tightness, wheezing and sputum production [6].

2.1.2 Pathogenesis

2.1.2.1 Inflammatory Cells

Asthma is a chronic inflammatory disease in which many cells are involved, including eosinophils, macrophages, mast cells, epithelial cells and lymphocytes (see Figure 2.1). The interactions of these cells result in the release of different mediators leading to bronchoconstriction, microvascular leakage, mucus hypersecretion, epithelial damage and stimulation of neural reflexes [3]. For example, macrophages products, such as IL-1 may prime eosinophils to respond to other secretory stimuli, and perhaps it is the

sequence of inflammatory cell interactions that makes the inflammatory response in asthma different from that of other conditions [7].

Several studies have reported that alveolar macrophages are key to the pathophysiology of several respiratory diseases including asthma. The activation of these cells induces the release of different inflammatory mediators: a) platelet-activating factor (PAF), a mediator that selectively attracts human eosinophils and causes eosinophil infiltration of airways and epithelial damage; b) gamma interferon, a powerful lymphokine which modulates lymphocyte and fibroblast function; c) tumor necrosis factor; d) interlukine 1 (IL-1), which activates lymphocytes and fibroblasts; e) antigen presentation to lymphocytes; f) alveolar macrophage derived growth factors for fibroblast etc. [3, 8-10].

Eosinophils and epithelial cells play an important role in the inflammatory process as well. Upon activation, eosinophils release a variety of mediators including leukotriene C_4, PAF, oxygen radicals and also basic proteins, which are toxic and lead to epithelial damage [11]. Epithelial cells release inflammatory mediators like LTB_4 and 15-lipoxygenase products which are chemotactic to other inflammatory cells [3].

2.1.2.2 Inflammatory mediators

Different mediators have been implicated in asthma, and they may have a variety of effects on the airways that account for most of the pathologic features of asthma (see Table 2.1). Mediators such as histamine, prostaglandins, and leukotrienes contract airway smooth muscle directly, increase airway mucous secretion, and attract and activate other inflammatory cells, which in turn release inflammatory mediators.

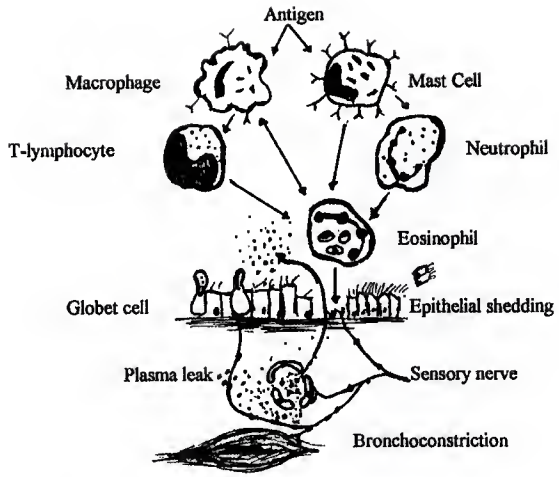

Figure 2.1 Cellular interactions. Adapted from reference [3].

2.1.2.3 The role of cytokines in allergic cell recruitment

As stated previously, asthma is characterized by the recruitment and migration of inflammatory cells, primarily eosinophils into the airways; cytokine generation in the airways may trigger such infiltration. It is believed that four classes of cytokines are important in allergic cell recruitment [12]: 1) non-specific endothelial activators (TNF-α and IL-1); 2) specific endothelial activators (IL-4 and IL-3); 3) those that activate eosinophil functions and prolong their survival (IL-3, IL-5, granulocyte-macrophage colony-stimulating factor (GM-CSF), and interferon gamma), and 4) those that directly stimulate cell migration (RANTES).

2.1.2.4 Neural mechanisms

Neural mechanisms (cholinergic, adrenergic and non-adrenergic-non-cholinergic) may be important in producing asthma symptoms (Figure 2.2). Factors that are under autonomic control include submucosal gland secretion, bronchial vascular tone and permeability, airway smooth muscle tone and probably secretion from inflammatory cells [3].

Table 2.1 Inflammatory mediators implicated in asthma [13].

Mediator	Broncho constriction	Airway secretion	Microvascular leakage	Chemotaxis	Bronchial hyperresponsiveness
Histamine	+	+	+	+	-
Prostaglandins D_2, F_{α}	++	+	?	?	+
Prostaglandin E_2	-	+	-	+	-
Leucotriene B_4	-	-	±	++	±
Leukotrienes C_4, D_4, E_4	++	++	++	?	±
Platelet activating factor	++	+	++	++	++
Bradykinin	+	+	++	-	-
Adenosine	+	?	?	?	-
Substance P	+	++	++	±	-
Serotonin	±	?	+	-	-
Oxygen radicals	+	?	+	-	-
Throboxane	++	?	-	?	+

2.1.3 Therapy of Asthma

The philosophy in the management of asthma has become the prevention and control of the disease analogous to the management of other chronic diseases such as hypertension [14]. In patients whose allergens are found at home or in the workplace the first priority is to prevent exposure or reduce it greatly, since continuous exposure can lead to chronic severe asthma that can no longer be controlled by avoiding the allergen [15]. What then is the best way to attenuate asthma?

Five classes of medication are effective in the treatment of asthma: anticholinergics, beta-adrenergic agonists, theophylline, sodium cromoglycate, and glucocorticoids [14]. Many controlled trials have now established that inhaled glucocorticoids are the most effective because of their effects at the cellular levels to attenuate the underlying disease process [16-19].

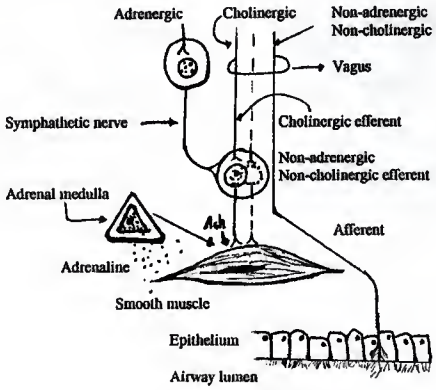

Figure 2.2. Neural mechanisms. Adapted from reference [3].

2.2 Glucocorticoids

Glucocorticoids consist of 21 carbon atoms with four rings, three six-carbon rings (A, B, and C), and a five carbon ring (D) (Figure 2.3). Most of the anti-inflammatory glucocorticoids are characterized by lipophilic susbtituents in positions 16 and 17; CH_3, F and Cl in positions 6 and 9; doble bound in carbons 1,2. Other essential features consist of the following: 1) a ketone oxygen at C-3, 2) an unsaturated bond between C-4 and C-5, 3) a hydroxyl group at C-11, and 4) a ketone oxygen at C-20. By modifying the basic structure of glucocorticoids, it appeared possible to modify the following functions [20-22]:

- Receptor binding: raising the affinity for the glucocorticoid receptor (GR), reducing the affinity for the mineralocorticoid receptor, and reducing the affinity for corticosteroid-binding globulin (CBG).
- Biotransformation: modulating the metabolism via oxido-reductive or hydrolytic pathways.
- Other physicochemical aspects: uptake, tissue binding and systemic disposition.

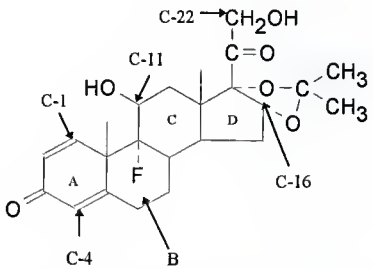

Figure 2.3 The chemical structure of glucocorticoids as typified by triamcinolone acetonide [23].

2.2.1 Molecular Mechanisms: the Glucocorticoid Receptor

The glucocorticoid receptor (GR; type II steroid receptors) belongs to the superfamily of steroid receptors. They represent transactivating proteins for genomic modulation. The glucocorticoid receptor is comprised of three domains: the ligand-binding, the DNA-binding and the modulatory domain [24]. The GR consists of approximately 780 amino-acids, with about 280 in the ligand-binding, 70 in the DNA-binding, and 430 in the modulatory domains. In its inactivated state, GR is complexed together with two molecules of heat shock protein 90-kD (HSP-90) and one molecule of the immunophilin p-59 (Figure 1.4). It is believed that these protcins cover the DNA-binding domain of the receptor [25] and that this complex does not show any biological activity.

When the glucocorticoid receptor is activated, it dissociates from the complex with HSP-90 and p-59 and exposes the DNA-binding domain with its two zinc fingers [26]. Two activated receptors will form a homodimer which is able to interact with specific DNA sequences called glucocorticoid response elements (GREs). Binding of the liganded GR to DNA usually results in activation of transcription through positive GREs (pGREs), but binding to less common so-called negative GREs (nGREs) can result in repression of transcription. The degree of the pharmacological effect is proportional to the degree of receptor occupancy. Such a correlation is one of the reasons for using receptor occupancy as a method to evaluate pulmonary targeting in our study

In recent years, it was found that GR can also modulate transcription through non-nuclear pathways. In this case, the activity of other transcription factors, such as AP-1 (activating protein-1) and NF-kB (nuclear factor kB), is modulated by direct interaction of

the activated glucocorticoid receptor with these two factors [27]. These newly discovered pathways are extremely important because they provide a cross-talk between glucocorticoids on one hand, and polypeptide hormones and cytokines on the other. Some of the genes regulated by this pathway include metalloproteinases, such as stromelysin and collagenase, and many cytokines, such as IL-2. The repression of such genes is likely to underlie the antiarthritic and immunosuppressive effects of glucocorticoids.

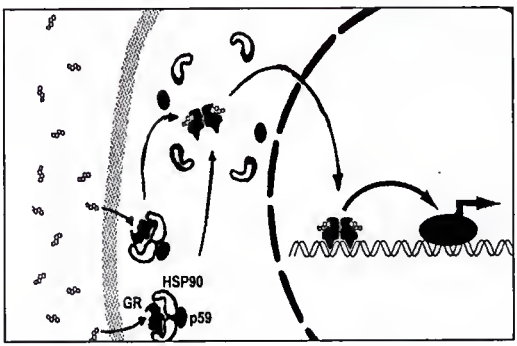

Figure 2.4. The Glucocorticoid receptor. Adapted from [18].

2.2.2 Target Genes in Asthma

The target genes involved in asthma are listed in Table 2.2. Glucocorticoids may be effective in controlling asthma by decreasing or increasing gene transcription.

Although it is not yet possible to be certain of the most critical aspects of steroid action in asthma, it is likely that their inhibitory effects on cytokine synthesis are of particular relevance [16]. Steroids inhibit the transcription of several cytokines that are relevant in asthma including IL-1, TNF α, granulocyte-macrophage colony-stimulating factor, IL-3, IL-4, IL-5, IL-6, and IL-8. Glucocorticoids increase the synthesis of lipocortin-1 a 37-KD protein that has an inhibitory effect on phospholipase A_2, and therefore may inhibit the production of lipid mediators such as leukotrienes, prostaglandins and platelet activating factor [28]. Glucocorticoids may be more effective in inhibiting cytokine release from alveolar macrophages than in inhibiting lipid mediators and reactive oxygen species [29].

2.2.3 Cellular Effects of Glucocorticoids

One of the best described actions of steroids in asthma is a reduction in circulating eosinophils which may reflect an action on eosinophil production in the bone. Inhalation of budesonide 800 μg twice a day produced a marked reduction in the number of low density eosinophils, presumably reflecting inhibition of cytokine production in the airways marrow [16]. Glucocorticoids are very effective in inhibiting the activation of lymphocytes and in blocking the release of cytokines, which are likely to play an important role in the recruitment and survival of several inflammatory cells involved in asthmatic inflammation.

While steroids do not appear to have a direct inhibitory effect on mediator release from lung mast cells, chronic steroid treatment is associated with a marked reduction in mucosal mast cell number. This may be linked to a reduction in IL-3 and stem cell factor (SCF) production, which are necessary for mast cell expression in tissues [19]. Epithelial

cells may be one of the most important targets for inhaled glucocorticoids in asthma. Glucocorticoids inhibit the increased transcription of the IL-8 gene induced by TNF-α in cultured human airway epithelial cells *in vitro*, the transcription of the RANTES gene and GM-CSF in an epithelial cell line [30].

Table 2.2 Effect of glucocorticoids on transcription of genes relevant to asthma [19].

Increased transcription

- Lipocortin-1
- β2-adrenergic receptor
- Endonucleasas
- Secretory leukocyte inhibitory protein
- Macrophage inhibitory factor

Decreased transcription

- Cytokines (IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-11, IL-12, IL-13, TNF-alpha, GM-CSF, RANTES,)
- Inducible nitric oxide synthase (iNOS)
- Inducible cyclo-oxygenase (COX-2)
- Inducible phospholipase A_2 ($cPLA_2$)
- Endothelin-1
- NK_1 receptors
- Adhesion molecules

2.2.3 Other Effects

Glucocorticoids exert a number of other effects:

- Immunosuppression
- Carbohydrate and protein metabolism (e.g. gluconeogenesis)
- Lipid metabolism (e.g. redistribution of body fat)
- Hypocalcemia
- Hypertension
- CNS effects (mood, behavior, neural excitability)
- Inhibition of cholesterol synthesis
- Cortisol suppression

2.3 The Pulmonary Route

Localized delivery of drugs to the respiratory tract has become an increasingly important and effective therapeutic method for treating a variety of pulmonary disorders including asthma, bronchitis, and cystic fibrosis. Although the traditional form of inhalation therapy dates back to the earliest records of ancient cultures, the advantages of inhalation therapy have essentially remained the same. Relatively small doses are required for effective therapy, reducing exposure of drug to the systemic circulation, and potentially minimizes adverse effects [31]. Lower dosage regimens may provide considerable cost saving especially with expensive therapeutic agents. Delivering small doses of active ingredients directly to the lung effectively localizes the drug, thereby maximizing therapeutic effect while minimizing unwanted side effects. As mentioned earlier, these advantages are the basis of inhalation therapy of glucocortioids.

2.3.1 Structure and Function of the Respiratory Tract

The respiratory tract can be divided into upper and lower airways with the line of division being the junction of the larynx and trachea [32]. The upper airways or nasopharyngeal region consists of the nose, mouth, larynx, and pharynx. Below the contours of the nasopharyngeal region, the lower airways resemble a series of tubes undergoing regular dichotomous branching [33]. Successive branching from the trachea to the alveoli reduces the diameter of the tubes, but markedly increases the surface area of the airways, which allows gas exchange. The lower airways can bc divided into three physiological zones: conducting, transitional, and respiratory zones [33] (see Figure 1.6). The conducting zone consists of the larger tubes responsible for the bulk movement of air

and blood. In the central airways, airflow is rapid and turbulent and no gas exchange occurs. The transitional zone plays a limited role in gas exchange. The characteristic D shape of the trachea is maintained by cartilage supported by smooth muscle fibers. The epithelial layer of the trachea and main bronchi are made up of several cell types including ciliated, basal, and globet. A large number of mucous- and serum-producing glands are located in the submucosa.

The human lung consists of five lobules and ten bronchopulmonary segments. Arranged to each segment are lung lobules composed of three to five terminal bronchioles. Each bronchiole supplies the smallest structural unit of the lung, the acinus, which consists of the alveolar ducts, alveolar sacs, and alveoli. At the level of small bronchi and bronchioles, the amount and organization of cartilage diminishes as the number of bronchial bifurcation increases. The acinus represents a marked change in morphology. The primary cells of the epithelium are the type I pneumocytes, which cover 90% of the entire alveolar surface. Type II pneumocytes are more numerous, but have a smaller total volume, and are responsible for the storage and secretion of lung surfactant. Less prevalent cell types include type III pneumocytes and alveolar macrophages. The alveolar blood barrier in its simplest form consists of a single epithelial cell, a basement membrane, and a single endothelial cell. While this morphological arrangement readily facilitates the exchange, it can still represent a major barrier to large molecules [34]. The lung tissue is highly vascularized, which makes pulmonary targeting difficult due to a fast absorption of most of the drugs (especially lipophilic and low molecular weight drugs).

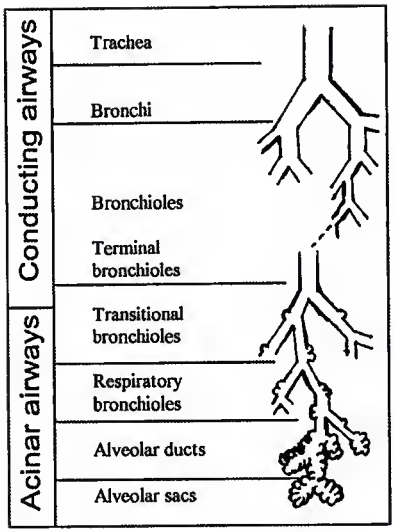

Figure 2.5 Organization of the airways. Adapted from reference [34].

2.3.2 Mechanisms of Drug Deposition

Drugs for inhalation therapy are administered in aerosol form. An aerosol is defined as a suspension of liquid or solid in the form of fine particles dispersed in a gas. A prerequisite for therapeutic efficacy is the ability of the aerosolized drug to reach the peripheral airways. Herein lies the fundamental problem of inhalation therapy as the anatomy and physiology of the respiratory tract has evolved to prevent the entry of particulate matter [34]. The regional pattern of deposition efficiency determines the

specific pathways and rate by which deposited particles are ultimately cleared and redistributed [35].

The mechanism by which particles may deposit in the respiratory tract are impaction (inertial deposition), sedimentation (gravitational deposition), Brownian diffusion, interception, and electrostatic precipitation [35]. The relative contribution of each depends on characteristics of the inhaled particles as well as on breathing patterns and respiratory tract anatomy.

2.3.2.1 Impaction

Impaction may occur when a particle's momentum prevents it from changing course in an area where there is a rapid change in the direction of bulk airflow. It is the main deposition mechanism in the upper airways, and at near bronchial branching points. The probability of impaction increases with increasing air velocity, rate of breathing, and particle size [35].

2.3.2.2 Sedimentation

Sedimentation results when the gravitational force on a particle is balanced by the sum forces due to the air resistance; inspired particles will then fall out of the air stream at a constant rate [36]. This is an important mechanism in small airways having low air velocity. The probability of sedimentation is proportional to residence time in the airway and to particle size, and decreases with increasing breathing rate.

2.3.2.3 Diffusion

The constant random collision of gas molecules with small aerosol particles pushes them about in an irregular fashion called Brownian movement. Thus, even in the absence of gravity, a particle in still air moves around in a "random walk" [36]. The effectiveness of Brownian motion is inversely proportional to particle diameters for those particles < 0.5 micron [37], and is important in bronchioles, alveoli, and at bronchial airway bifurcation. Molecular-sized particles may deposit by diffusion in the upper respiratory tract, trachea, and larger bronchi.

2.3.3 Factors Controlling the Fate of Aerosols in the Respiratory Tract

The fate of inhaled particulates in the respiratory tract and therefore, the local therapeutic activity, depends upon the dynamic interaction of different factors: 1) deposition, 2) mucociliary clearance, 3) drug dissolution rate or release, 4) absorption, 5) tissue sequestration, and 6) metabolism kinetics. Extensive literature exists on the theoretical and experimental aspects of each of these factors [35, 36, 38-41].

2.3.3.1 Factors controlling respiratory drug deposition

The factors that control drug deposition are 1) characteristics of the inhaled particles such as size, distribution, shape, electrical charge, density, and hygroscopicity; 2) anatomy of the respiratory tract, and, 3) breathing patterns such as frequency, depth, and flow rate.

2.3.3.1.1 Particle characteristics

The particle size is a critical factor affecting the site of their deposition, since it determines operating mechanisms and extent of penetration into the lungs. Aerosol size is often expressed in terms of aerodynamic diameter (D_{ae}). Aerodynamic diameter is defined as the diameter of a spherical particle having unit density that has the same settling velocity from an air stream as the particle in question [35]. Thus particles that have higher than unit density will have actual diameters smaller than their D_{ae}. An aerosol has a size distribution arbitrarily characterized as monodispersed (uniform size distribution and geometric standard deviation of < 1.2) or polydispersed (less uniform size distribution and geometric standard deviation equal to or > 1.2).

2.3.3.1.2 Respiratory tract anatomy

In air-breathing animals, respiratory anatomy has evolved in such a way as to actively thwart inhalation of putative airborne particulates [42]. The upper airways (nose, mouth, larynx and pharynx) and the branching anatomy of the tracheobronchial tree act as a series of filters for inhaled particles. Thus, aerosol particles, whose diameter is greater than 100 micron, generally do not enter the respiratory tract and are trapped in the naso-oropharynx. Particles larger than 10 micron will not penetrate the tracheobronchial tree. Particles must generally be less than 5 micron in order to reach the alveolar space [36]. On the other hand, particles of 0.5 micron in diameter will penetrate the lung deeply, but have a high tendency to be exhaled without deposition.

Airway geometry affects particle deposition in various ways. For example, the diameter sets the necessary displacement by the particle before it contacts an airway

surface, cross-section determines the air velocity for a given flow rate, and variations in diameter and branching patterns affect mixing between tidal and reserve air [35]. Thus, since the respiratory system of animals and humans differs anatomically, differences in deposition patterns are also expected.

2.3.3.1.3 Respiratory patterns

The pattern of respiration during aerosol exposure influences regional deposition, since breathing volume and frequency determine the mean flow rates in each region of the respiratory tract which, in turn, influence the effectiveness of each deposition mechanism. Turbulence tends to enhance particle deposition, with the degree of potentiation depending of the particle size [35]. Rapid breathing is often associated with increased deposition of larger particles in the upper respiratory tract, while slow, steady inhalation increases the number of particles able to penetrate to the peripheral parts of the lungs [43, 44]. Byron (1986) [39] developed a mathematical model which allowed him to identified the effect of particle size and breathing pattern on lung drug deposition. Slow breathing, with or without breath-holding, showed broad maximum deposition in the ciliated airways (tracheobronchial portion TB). The pulmonary maximum occurs between 1.5-2.5 and 2.5-4 micron, with and without breath-holding, respectively. Rapid inhalation showed similar trends; the TB maximum falls and shifts to between 3 and 6 micron. Pulmonary deposition sharpens and occurs between 1.5-2 and 2-3 microns, with and without breath-holding, respectively.

When the above considerations are taken into account, the ideal scenario for an aerosol would be 1) aerosol D_{ae} < 5 micron to minimize oropharyngeal deposition, 2) slow, steady inhalation, and 3) a period of breath-holding on completion of inhalation.

2.3.3.2 Pulmonary clearance mechanisms

Insoluble particulates are cleared by several pathways which today are only partially understood. These pathways are known to be impaired in certain diseases and are thought to depend on the nature of the administered material [45]. Swallowing, expectoration, and coughing constitute the first sequence of clearance mechanisms operand in the naso-oropharynx and tracheobronchial tree [42]. A major clearance mechanism for inhaled particulate matter is the mucociliary escalator. It consists of ciliated epithelial cells reaching from the naso-oropharynx and the upper tracheobronchial region down to the most peripheral terminal bronchioles. Beating of the cilia, together with mucus secreted by the globet cells, contributes to an efficient clearance mechanism. Mucociliary clearance kinetics are difficult to quantify. Studies of clearance kinetics usually involve administration of 3.32 micron (D_{ae}) insoluble iron oxide aerosol to normal humans. Byron (1986) [39], using a mathematical model, analyzed the clearance data resulting from the administration of iron oxide. He found that 48.9 % of the deposited material remained 24 h following administration. The major clearance mechanism in the alveolar regions of the lung is uptake by alveolar macrophages [42].

2.3.3.3 Absorption kinetics

The rate at which drugs are cleared from the airways depends greatly on certain physicochemical properties of the drug, such as 1) molecular weight, 2) partition coefficient and, 3) binding characteristics.

Enna and Shancker [46] have examined the lung absorption of saccharides and urea of various molecular weights. They found that the first-order rate constant, k, decreased with increasing molecular weight. Because of the low lipid solubility of these hydrophilic compounds, these investigators proposed that absorption occurred through aqueous channels of intercellular spaces in the lipid membrane. In another study, it was found that those compounds with molecular weights less than 1000 D were absorbed at faster rates ($t_{1/2}$ = 90 min) than the larger molecules ($t_{1/2}$ = 3 to 27 h). First order absorption rate constants from the upper respiratory tract are approximately half of those from the alveolar regions [40]. Solutes with molecular weights in the range of 100 to 1000 D are absorbed with half-lives between 8 and 40 min [47].

Some studies have reported that solutes penetrate the alveolar wall at rates which increase with the lipid solubility of the compound [48]. Those compounds having partition coefficients greater than 10^8 are absorbed rapidly. However, there appears to be no continuity for others with partition coefficients less than 10^6 [49]. If the partition coefficients and molecular weights of the compounds are taken into consideration simultaneously, it may be possible to estimate which compound will be absorbed most quickly.

2.3.3.4 Drug dissolution or release

It has been reported that the physicochemical properties of the drug such as particle size (surface area) and crystal form [50], as well as its lipophilicity [51], play a central role in the dissolution rate. Drugs with higher lipophilicity and larger particle size will dissolve slower. A drug with a fast release rate will be dissolved immediately after pulmonary deposition and will be absorbed into the systemic circulation at a rate which will be determined by its partition coefficient and molecular weight as mentioned earlier in section 2.3.3.3. See also section below for the effect of release rate on pulmonary targeting.

2.4 Pulmonary Targeting

The purpose of pulmonary targeting is the achievement of relatively high drug lung concentrations relative to drug systemic concentrations, thereby reducing systemic side effects.

2.4.1 Pulmonary Selectivity of Inhaled Glucocorticoids

Inhaled glucocorticoids (IG) were first introduced into asthma therapy to replace oral glucocorticoids in patients with severe asthma. The advantage of inhalation therapy versus oral therapy is the direct delivery to the lungs, combined with a relatively lower dose, which may reduce the incidence of systemic side effects [52].

Beclomethasone dipropionate, triamcinolone acetonide, flunisolide, budesonide and fluticasone propionate are currently available as aerosols in the US for the treatment

of asthma [51-53]. They are delivered by metered dose inhalers (with and without spacers), dry powder inhalers and nebulizers.

Due to the apparent uniformity of the glucocorticoid receptor in the body, there was no basis for differentiation of desirable from undesirable glucocorticoid effects at the receptor level. However, through chemical manipulation of the glucocorticoid structure it has been possible to develop synthetic derivatives better suited for pulmonary targeting. Thus, glucocorticoids with lipophilic substituents in the 17α or 16α, 17α positions were found to be better candidates for inhalation therapy, especially because of the improvement of pharmacokinetic properties.

Although the pharmacokinetic properties of inhaled glucocorticoids favor its pulmonary selectivity, one problem associated with the respiratory drug delivery is the relatively rapid absorption (once they are dissolved) into the systemic circulation requiring frequent administration. Since frequent administration of these drugs is associated with high risk of systemic side effects (especially suppression of growth in children and osteoporosis), current research is aimed at identifying the factors that can improve pulmonary targeting is obvious.

Hochhaus et al. [1] have developed a pharmacokinetic/pharmacodynamic model to aid prediction of the pharmacokinetic and biopharmaceutical factors which affect pulmonary targeting. Contrary to previous mathematical models [39, 54], this model integrated lung physiology with pharmacokinetic, pharmacodynamic and biopharmaceutical drug properties, and thereby allowed prediction of pulmonary and systemic effects by calculating pulmonary and systemic receptor occupancies, as

pharmacodynamic surrogate. This model, which gave the basis for the *in vivo* studies presented in this thesis, is described below using glucocorticoids as a model.

2.4.1.1 Effect of clearance, volume of distribution and oral bioavailability

Commercially available glucocorticoids differ in their clearance and oral bioavailability. Oral glucocorticoids are characterized by a low clearance and high oral bioavailability, while inhaled glucocorticoids have a high clearance and low oral bioavailability (see Table 2.3). Inhaled glucocorticoids also differ in their volume of distribution (Table 2.3).

The computer simulations described above showed that an increase in clearance (from 6 L/hr to 60 L/hr) resulted in a dramatic decrease in systemic side effects from 61.3% to 12.4%, with a small decrease in pulmonary effects. However, the difference between pulmonary and systemic effects increased, and as a consequence, so did pulmonary targeting (defined in here as the ratio of pulmonary effects and systemic effects). In contrast, changes in volume of distribution did not seem to significantly affect pulmonary targeting. Thus, drugs with high systemic clearance are better candidates for attaining pulmonary selectivity.

Only 10% to 30% of the delivered drug is deposited along the airways. The rest is swallowed and absorbed into the systemic circulation (see Figure 2.6). The percentage of systemically absorbed drug that is related to the oral route depends on the glucocorticoid oral bioavailability.

Table 2.3 Pharmacokinetic and pharmacodynamic parameters of commercially available glucocorticoids [1].

	Clearance (L/hr)	Volume of distribution (Vdss) (L)	Half-life (hr)	Oral bioavailability (%)	Plasma binding (%)	RBA
Dexamethasone	16.8	100	4.5	80	77	100
Triamcinolone acetonide	37	103	2	23	71	233
Flunisolide	58	96	1.6	20	80	190
Budesonide	84	183	2.8	11	88	935
Fluticasone propionate	69	318	7.8	<1	90	1800

Thus drugs with low oral bioavailability will be a better drugs for inhalation therapy. Since inhaled glucocorticoids differ in their oral bioavailability (Table 2.4) it may be interpreted as differences in pulmonary selectivity.

2.4.1.2 Effect of pharmacodynamics

It has been stated that only glucocorticoids with high relative binding affinity (RBA) for the glucocorticoid receptor are active by inhalation, and that the minimum level of RBA seems to be 20 times above of hydrocortisone [55]. In addition Brattsand et al. [23], have mentioned that a high affinity for and intrinsic activity at the glucocorticoid receptor seems necessary for gaining topical anti-inflammatory activity on the mucous

membranes. However, the pk/pd simulations [1] described above, have demonstrated that a low receptor affinity can be compensated by an increase in dose, assuming that the required dose can be delivered via inhalation. Thus, this suggests that glucocorticoids with high receptor affinity may not be better drugs to enhance pulmonary targeting.

2.4.1.3 Effect of release rate and dose

Glucocorticoids are removed relatively fast from the airways (once they have been dissolved) due to their relatively high lipophilicity and because of the high pulmonary blood flow. Hochhaus et al. [1] have theoretically demonstrated the relevance of release rate on pulmonary selectivity. He showed that a glucocorticoid with fast dissolution rate will be removed immediately from the airways by entering the systemic circulation (see Figure 2.6), resulting in small mean residence time values. After a short time, the free drug concentrations will be the same in lung and systemic organs, and minimal pulmonary targeting will be observed. If the release rate decreases, the free drug concentrations in lung will be higher over an extended period of time than that in the systemic organs, resulting in more pronounced pulmonary effects and unchanged systemic effects. Thus, it was concluded that sustained release rate is very beneficial for improving lung selectivity. However, the author mentioned that for drugs deposited into the upper portion of the airways, a further reduction in drug release will result in reduction in pulmonary targeting due to elimination of the undissolved drug by mucociliary transport. Likewise, he showed that when the dose is too small it could mean that the minimum effect concentration is not reached either in the local organ (lung) or in the systemic organ (liver). When the dose is

too high toxic side-effects may result and pulmonary targeting is lost. Thus, there is an optimum dose and release rate for which maximum pulmonary targeting can be achieved.

Some studies [23, 56] have suggested the importance of drug release rate in pulmonary targeting. In these studies the intratracheal administration of glucocorticoids in slow release preparations yielded higher pulmonary selectivity than that after the intratracheal administration of the drugs in solution.

2.4.2 Improvement of Glucocorticoid Airway Selectivity

Although the benefits of inhaled over oral glucocorticoids are clear, there are still some concerns regarding side effects since inhaled glucocorticoids are likely to be administered over long periods of time. This issue requires new emerging technologies that can lead to the enhancement of airway selectivity. For example, the design of glucocorticoids with extra-hepatic metabolism will lead to derivatives with even higher clearance and reduced systemic side effects; the use of drug delivery systems such as liposomes, or new drug entities will increase the pulmonary residence time and thus, more prolonged pulmonary effects. This thesis proposes the use of liposomes as a slow drug delivery system to improve pulmonary selectivity

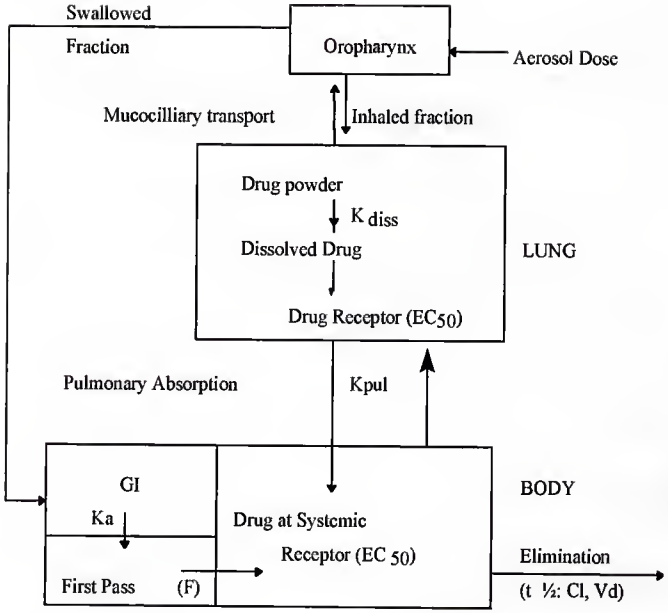

Figure 2.6 Pharmacokinetic/Pharmacodynamic model for pulmonary selectivity [1].

2.5 Liposomes

In the last two decades, it has been demonstrated that encapsulation of drugs into liposomes can lead to the enhancement of therapeutic efficacy of drugs, reduction of their toxicity and prolongation of their therapeutic effect. In addition, studies have been performed on the use of liposome-based vaccines. Recently, a liposome formulation containing the antifungal agent amphotericin has been approved and is presently used in the clinic [57] . This suggests that the spirit and enthusiasm to explore new research

strategies with liposomes are as alive now as they were in the early days of liposome research.

Since selective drug delivery to specific tissues is the main application of liposomes, efforts have been principally concentrated on "site-directed targeting," for instance, selective uptake. As a result of these studies, a detailed picture of the tissue distribution of systematically administered liposomes has emerged [58]. However, the kinetics of the disposition processes are still far from being characterized.

Liposomes are quite similar to natural membranes, and are thus inert and biodegradable. Through variation in size, lipid composition, number of bilayers, charge and surface characteristics, the pharmacokinetics of liposomes and therefore the pharmacokinetics of the encapsulated drug, can be manipulated.

2.5.1 Liposome Structure and Classification

Liposomes are vesicular structures composed usually of phospholipid bilayers which can entrap hydrophilic or hydrophobic materials within their aqueous compartment or within the membrane. These vesicles are formed spontaneously when lipids are dispersed in aqueous media. Depending on the method of preparation, the population of vesicles can range in size from the smallest vesicles obtainable in theoretical grounds (25 nm in diameter), determined by the maximum possible crowding that headgroups will tolerate as the curvature in the inner leaflet increases with decreasing radius, to liposomes which are visible under a light microscope with a diameter of 1 micron [59].

Liposomes can be classified either on the basis of their structural properties or on the basis of the preparation method used. Table 2.4 lists the different vesicles with the corresponding acronyms.

2.5.2 Chemical Composition.

Liposome-forming phospholipids are obtained from natural sources or through synthetic routes. Natural phospholipids are not easy to characterize in terms of their exact phospholipid composition. In contrast, synthetic phospholipids can be well characterized and can be obtained in highly purified forms [59]. Two sorts of natural phospholipids exist: phosphodiglycerides and sphingolipids. In phosphodiglycerides, a three-carbon glycerol bridge links a long chain fatty acid with a phosphoryl headgroup moiety; by convention, the fatty acids are said to occupy positions 1 and 2 of the glycerol bridge while the polar headgroup is in position 3.

Choline-containing phospholipids, also known as lecithin, are the most abundant phosphodiglycerides in nature. They form the major phospholipid component of many cell membranes. Because of their neutrality and chemical inertness, they are the most often used lipids for the preparation of liposomes. The chemical structure of a number of phospholipids regularly used for liposome preparation is show in Figure 2.6.

Table 2.4 Liposome Classification [60]

A. Based on Structural parameters	
MLV	Multilamellar large vesicles, > 0.5 μm
OLV	Oligolamellar vesicles, 0.1-1 μm
UV	Unilamellar vesicles (all sizes)
SUV	Small unilamellar vesicles, 20-100 μm
MUV	Medium-sized unilamellar vesicles
LUV	Large unilamellar vesicles, > 100μm
GUV	Giant unilamellar vesicles (vesicles with diameters > 1 mm)
MVV	Multivesicular vesicles (usually large > 1 mm)
B. Based on Method of Liposome Preparation	
REV	Single or oligolamellar vesicles made by reverse-phase evaporation method
MLV-REV	Multilamellar vesicles made by reverse phase evaporation method
SPLV	Stable plurilamellar vesicles
FATMLV	Frozen and thawed MLV
VET	Vesicles prepared by extrusion methods
FPV	Vesicles prepared by French press
FUV	Vesicles prepared by fusion
DRV	Dehydration-rehydration vesicles
BSV	Bubblesomes

2.5.3 Method of Preparation

Liposomes are prepared by mechanical dispersion methods (such as hand-shaken multilamellar vesicles, non-shaken vesicles, freeze-drying, sonicated vesicles methods, etc), by solvent dispersion methods (such as ethanol injection, ether injection or water in organic phase method) or by a detergent solubilization method [60].

Dependent on the selection of lipids, the preparation technique, and preparation conditions, liposomes can vary widely in size, number, position of lamellae, charge, and bilayer rigidity. These parameters influence the behavior of liposomes both *in vivo* and *in vitro*. The opsonization process, leakage profiles, disposition in the body, and shelf life all depend on the type of liposome involved [60]. Therefore, it is important to select liposome

constituents and the preparation technique carefully and to characterize the produced liposomes properly.

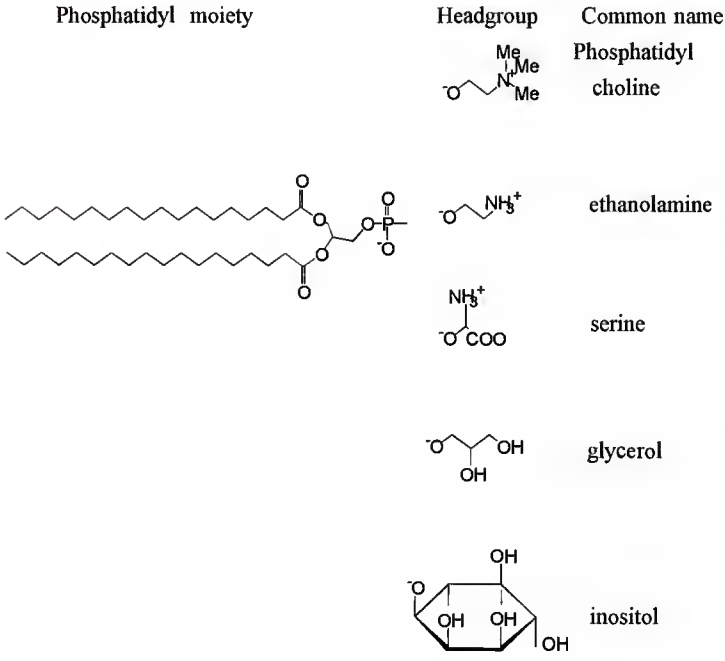

Figure 2.6 Some common naturally-occurring phosphatidyl phospholipids. Adapted from reference [57]

2.5.4 Chemical Stability

From a pharmaceutical point of view, it is important to demonstrate that drugs or dosage forms are sufficiently stable, so that they can be stored for a reasonable period of time (at least 1 year and preferably) without changing into inactive or toxic forms. Liposomal drug formulations also must comply with this rule.

The chemical stability of liposomes is focused mainly on phosphatidylcholine, because as previously mentioned, it is the most commonly used lipid in pharmaceutical liposome preparations. Two degradation pathways which might limit the shelf life of liposome dispersions have been described for phospholipids in aqueous liposome dispersions. These are the oxidative and the hydrolytic degradation pathways [57].

2.5.4.1 Oxidation of Phospholipids

Oxidation of phospholipids in liposomes mainly takes place at the unsaturated acyl chain-carrying phospholipids, but saturated fatty acids can also be oxidized at high temperatures [57].

The fatty acyl chains of phospholipid molecules are oxidized via a free radical chain mechanism in the absence of specific oxidants. Exposure to electromagnetic radiation and/or the presence of trace amounts of transition metal ions, initiate radical formation (hydrogen atom abstraction) in the lipid chain (initial step). Since unsaturation permits delocalization of the remaining unpaired electron along the lipid chain, lipids containing polyunsaturated fatty acids are the most sensitive to radical formation. If oxygen is present, the process develops further and via the formation of hydroperoxides, fission of

the fatty acid chain can occur. Changes in UV absorbance of lipids is the first sign of the occurrence of radical chain reactions which can lead to oxidation [60].

As mentioned above, the autoxidation of lipids is accelerated by metal ions, light, some organic molecules and also by high pH. It is clear then, that this oxidation process can be minimized by the use of high quality raw materials which are purified from hydroperoxides and transition metal ions, storage at low temperatures and the use of antioxidants.

Gonzalez-Rothi et al. [61] evaluated the *in vitro* stability of various formulations of liposomes is several fluids. They found that liposomes consisting of unsaturated phospholipids, lost around 40% of their content over 24h compared to liposomes made of saturated phospholipids which retained more than 90% of the encapsulated material. It is possible that the increase in permeability in the unsaturated formulation was due to a higher degree of autoxidation process in relation to the saturated formulations.

2.5.4.2 Hydrolysis of phospholipids

The liposome is inherently a stable system. In the absence of oxygen and free radicals the only chemical reactions which result in a change in liposome properties are those of hydrolysis.

The four ester bonds present in a phospholipid molecule may be subject to hydrolysis. The carboxy esters at sn-1 and sn-2 positions are hydrolyzed faster than the phosphate esters.

Hydrolysis of esters is catalyzed via two pathways [57]:

- Acid catalyzed hydrolysis. In this pathway the ester is first protonated and subsequently, a nucleophilic attack of water takes place.

- Base catalyzed hydrolysis. This step proceeds via an addition-elimination mechanism. Here the rate determining step is the attack of hydroxide ions on the carbonyl carbon atom.

Two products are formed as a result of hydrolysis of the carboxy esters at the sn-1 and sn-2 positions: 2-acyl and 1-acyl lysophospholipids, respectively [62]. Further hydrolysis of both lysophospholipids produces a glycerophospho compound. Glycerophosphoric acid, the end hydrolysis product, is produced by hydrolysis of the phosphate-headgroup ester.

The rate of hydrolysis can be affected by different factors:

- pH
- temperature
- ionic strength
- buffer species
- chain length
- incorporation of cholesterol
- incorporation of charged phospholipids

2.5.4.2.1 The effect of pH

Grit et al. [63], studied the effect of pH on the hydrolysis of saturated soybean phosphatidylcholine (PC) in a pH range from 4-9 at 40 °C and 70 °C. They found that the hydrolysis rate reached a minimum at pH 6.5, and V-shaped pH profiles were obtained as expected for an acid/base catalyzed ester hydrolysis. In another study the pH profile at zero buffer concentration and at 72 °C showed a minimum hydrolysis rate at about pH 6.5 for natural soybean PC [64].

2.5.4.2.2 The effect of temperature

The effect of temperature on the hydrolysis of phospholipids has been studied with synthetic and/or natural PC. For all PC's the results could be described by Arrhenius kinetics as a semilogarithmic linear relationship was found between the observed hydrolysis rate constant and the reciprocal of the absolute temperature. For natural PC's, Arrhenius kinetics held in a temperature range from room temperature up to 82 °C [64]. However, for phospholipids composed of saturated fatty acids and with a liquid to gel crystalline phase transition, biphasic Arrhenius curves were obtained with a discontinuity around the transition temperature [62].

2.5.4.2.3 The effect of charged liposomes

Charged phospholipids are generally included in pharmaceutical liposome formulations as they tend to improve the physical stability of liposomes by reducing the rate of aggregation and fusion. Recently, hydrolysis of partially hydrogenated egg PC and egg phosphatidylglycerol (PG) have been investigated under different conditions [57]. Different electrostatic profiles in the aqueous phase around the bilayers were obtained by varying the charged phospholipid content (PG) of liposomes. Hydrolysis kinetics for both lipids followed pseudo first order reaction kinetics. It was found that egg PG hydrolyses faster than partially hydrogenated egg PC.

2.5.4.2.4 The effect of incorporation of cholesterol

The incorporation of cholesterol into liposomal bilayers tends to increase the retention of hydrophilic drugs, counteracts lipid phase transition and increases the rigidity

of fluid state bilayers, and thereby, the resistance to liposome degradation. The effect of cholesterol on the properties of distearoylphosphatidylcholine (DPPC) vesicles was studied using a fluorescence polarization technique by Papahadjopoulos et al. [65]. They found that cholesterol abolishes the phase transition of phospholipids, producing a rather rigid membrane over a wide temperature range, which is translated into a protective effect against hydrolysis.

Based on the structural properties of phospholipids and cholesterol molecules, Huang C. [66], proposed a model for the effects of cholesterol on the structural and motional properties of phospholipid acyl chains. In this model, the 3β-hydroxy group of cholesterol is assumed to engage in hydrogen bonding with the carbonyl oxygen of the fatty acyl groups in phospholipids. Depending on the strength of this interaction, the hydration properties of both ester bond (sn-1 and sn-2) may change, which in turn can change the hydrolysis kinetics. This effect is, in general, expected to be a protective effect, as mentioned above.

2.5.5 Physical Stability

Chemical decomposition of phospholipids causes physical instability of the liposome dispersion, which means that all the variables considered in chemical stability will affect the physical stability of liposomes and therefore might interfere with the introduction of liposomes in therapy.

The physical stability of liposomes is influenced by two parameters:

1. Changes in the average particle size and size distribution due to vesicle aggregation and fusion.
2. Loss of entrapped drug due to leakage.

Changes in the average particle size and size distribution are strongly affected by:

- Phospholipid composition
- Medium composition

2.5.5.1 The effect of phospholipid composition

Liposomes which lack a net electrical charge tend to be less stable towards aggregation than charged liposomes. Crommelin [67] studied the effect of including charge-inducing agents (SA and PS) on the zeta potential of PC containing liposomes in aqueous media with varying ionic strength. It was found that for negatively charged liposomes, both at low and high ionic strength, no increase in particle size occurred after storage at 4°C for 14 days. Thus, aggregation can be prevented or slowed down by incorporation of a charge-carrying lipid into the liposomal formulation.

2.4.5.2 Liposome composition

Several studies have shown that the incorporation of cholesterol into liposomes increases the physical stability of the formulations by increasing rigidity of the bilayer which is translated in decreased permeability [65, 66, 68-71].

As mentioned above, lysophospholipids and free fatty acids are produced as a result of phospholipid hydrolysis. These products have different amphiphilic properties

than the parent drugs and can produce morphological changes in the membrane and thus changes in permeability of the bilayer [68-70].

The effect of lysophospholipids and fatty acids formed *in situ* was investigated [57]. Liposomal dispersions were stored over time intervals at elevated temperatures to produce degradation of the phospholipids. It was found that when up to 10% lysophosphatidylcholine (LPC) was produced (corresponding to 10 % of hydrolysis), a drop in the leak-in rate of calcein occurred. Above that degree of hydrolysis, the leak-in rate started to increase as in exogenous LPC containing liposomes. They concluded that in the initial stage the free fatty acids were also formed, thus, neutralizing the LPC-destabilizing effect.

2.5.5.3 Liposome size

The effect of vesicle size on the physical stability of liposomes depends on the lipid composition and the medium considered.

Large neutral liposomes are less stable than smaller liposomes, because the increased planarity of the membranes allows greater areas of membrane to come into contact (Van der Waals interactions) with each other, and an increase in aggregation produces an increase in permeability.

Small negatively charged unilamellar vesicles (40 nm) are less stable (thermodynamically) than large vesicles. They fuse easily as a means of relieving stress arising from the high curvature of the membrane [60]. In general, large multilamellar liposomes are more stable against leakage than small unilamellar vesicles due to the difference in membrane thickness.

Size-dependent stability of liposomes in biological fluids has been reported by different authors. Tari et al. [72] studied the relationship between liposome size and its stability in plasma. They found that small liposomes composed of pure succinylglycerol were more stable than larger ones. However, in another study [71], it was found that small liposomes consisting of egg phosphatidylcholine and cholesterol were less stable than larger liposomes. As can be seen from these results stability of charged and neutral liposomes with respect to the size is completely different (inverse) to that in buffers. This may be due to a different degree of protein association on the surface of the bilayer, or that the final concentration of plasma used in either case was different. It has been shown [73] that at low serum concentrations liposome formulations consisting of DPPC, PI, SA and cholesterol remain intact independently of size, but as the serum concentration is decreased, the size of the liposomes decreases with concomitant release of encapsulated material.

2.5.5.4 Presence of metal ions

Divalent cations besides enhancing fusion and aggregation of liposomes as mentioned above, can cause an increase in the permeability of the bilayer.

The interaction of liposome bilayers composed of succinylglycerol with divalent ions was investigated by Tari, et al. [72]. In this study, it was found that Ca^{2+} and Mg^{2+} produce a destabilization of the bilayer due to an isothermic phase change of the lipid bilayer from liquid to a crystalline state which resulted in increased permeability of the encapsulated material.

2.5.6 Biological Stability

When administered *in vivo,* liposomes are rapidly cleared by the reticular endothelial system (RES), in such a way that large, negative liposomes consisting of unsaturated phospholipids are taken faster than small, neutral liposomes.

Advances in therapeutic applications of liposomes have been achieved through surface modifications increasing their biological stability, thus, reducing constituent exchange and leakage as well as reducing unwanted uptake by cells of the RES.

Perhaps one of the earliest descriptions of liposomes exhibiting a substantially prolonged circulation was of small, neutral, cholesterol-rich liposomes composed of phospholipids with a high temperature phase transition. However, severe limitations to a small particle and neutral rigid lipids in combination with a lipid dose-dependence reduces their therapeutic usefulness [74]. Subsequent incorporation of specific natural glycolipids such as monosalo-ganglioside (G_{M1}) or hydrogenated soy phosphatidylinositol was shown to result in prolonged circulation and reduced RES uptake.

2.5.7 Pharmacokinetics of Liposomes

In contrast to more common drugs which form true solutions in body fluids, phospholipid liposomes are colloidal suspensions. Accordingly, they are cleared from the blood by mechanisms different from those governing the clearance of substances dispersed at the molecular level. This has prevented the use of the pharmacokinetic models currently applied to the analysis of the disposition kinetics of drugs. Although, some kinetic models have been proposed to describe the disposition of certain types of liposomes, a model of

general applicability is still lacking and a quantitative analysis of liposome disposition is hardy feasible [75] .

The fate of liposomes administered to the body is drastically altered by administration route, dose, size, formulation, and surface charge, and specific liposomal surface properties modified by certain ligands, sugars, antibodies, etc. [76] .

2.5.7.1 Disposition of liposomes following intravenous (IV) administration

Liposomes administered IV can interact either with phagocytic cells (fusion or endocytosis) or with plasma lipoproteins. Liposomes, similar to any foreign particulate matter, are rapidly removed from the circulation mainly by the Kupffer cells, and to a lesser extent, by the other macrophage populations. The RES represents, therefore, a major obstacle to an effective targeting of liposomes to other cell types.

The rate and uptake of liposomes by the RES depend on liposome size, physical state, composition, and dosage [75]. Therefore, since the RES is the principal mechanism for liposome clearance, liposome clearance will be affected by changing dose, chemical composition etc.,

Internalization of phospholipid liposomes is also operated by non-phagocytic cells such as fibroblasts, cells of the kidney, lymphocytes, hepatocytes, etc. This non receptor-mediated endocytosis appears to be strictly dependent on the size of liposomes, with the optimal size being 50-100 nm. Vesicles larger than 400 nm are not apt to be endocytosed [75].

Phospholipid vesicles can interact with, and become destabilized by plasma lipoproteins, mainly high density lipoproteins (HDL). This destabilization is the result of

the insertion of apoproteins into the liposomal bilayer and the exchange of lipidic material between lipoproteins and liposomes [75]. The degree of liposomal destabilization, and therefore, the biological stability and half-life depend again, on the size and composition of the vesicles.

The size of the endothelial cells (about 70 nm in diameter) does not permit accommodation of particles larger than about 50 nm, therefore phospholipid liposomes are unable to leave the vascular space by passing across the cells of the capillary endothelium. Thus, egress of liposomes from the circulation is dependent on the presence of pores like those in fenestrated or discontinuous capillaries present in the liver, and appears a realistic possibility only for SUV [77].

2.4.7.1 1 Effect of vesicle size on the kinetics of liposomes

Small unilamellar vesicles (< than 100 nm) are taken up less rapidly than large multilamellar vesicles and when those small liposomes go to the liver, there is a higher proportion entering the hepatocytes rather than the Kupffer cells, since they are small enough to pass through the fenestrae of the sinusoids and into the liver parenchyma. Accordingly, SUV encounter longer half-lives (days) and smaller clearance than LMV [75].

As noted above, liposomes are destabilized upon IV administration due to unidirectional transport of phospholipids from the liposomes to HDL. Resistance to such a disruption depends on the diameter of the vesicles; large liposomes are less readily penetrated by apoproteins than are SUV and, therefore, exhibit greater stability.

A biphasic plasma decay curve is quite often observed following IV injection of liposomes, with a faster initial decay followed by a slower one. For substances that form true solutions in body fluids, such a profile is analyzed on the basis of a multicompartmental open model.

Huang et al. [78], observed a biphasic decay and extrapolated the volume of distribution to be 1.28 times larger than the volume occupied by the erythrocytes after the IV administration of SUV (19 nm mean diameter) prepared from sphingomyelin and cholesterol. Since the ratio of the volume of distribution of the central compartment (Vc) to the extrapolated volume of distribution (Vd) was 0.78, they concluded that a 1 compartment body model was completely satisfactory for the disposition kinetics of this liposome preparation. The value of the Vc/Vd ratio, the limits of which are 0 to 1, is indicative of the multicomparmental character of a disposition kinetics. The smaller the numerical value of the ration, the greater the multicompartmental character.

As mentioned above, large liposomes do not distribute outside the plasma compartment. If egress from plasma is unidirectional, then it is an elimination process (irreversible loss of the substance from the site of measurement) that cannot be responsible for a biphasic decay. Then, what factors are responsible for this biphasic decay? This question has been addressed by several investigators [79, 80].

Juliano and Stamp [79], by showing that small vesicles are cleared at a slower rate than large vesicles, demonstrated that a biphasic plasma decay may result from heterogeneity in size of the injected liposomes. They found that a biexponential clearance pattern is observed with heterogeneous vesicles populations, whereas a monoexponential decay is seen with liposome samples homogeneous in size.

Gregoriadis et al. [80], observed that the biphasic decay pattern of IV injected MLV is converted into a linear one upon increasing the liposomes dose. They suggested that the biphasic decline may be due to the presence of two elimination pathways: a faster one, due to a saturable uptake by the RES, and a slower one, due to uptake by the liver parenchymal cells. When the retention capacity of the RES becomes saturated, then only the slower elimination pathway operates and the decay rate decreases.

2.5.7.1.2 Effect of lipid composition and surface charge on the kinetics of liposomes

Upon exposure to blood, liposomes become coated (opsonized) with plasma proteins (alpha 2-macroglobulin) that can mediate their uptake. Recognition and ingestion of opsonized liposomes by phagocytic cells are mediated via binding of liposomal opsonins to specific receptors on these cells. A major mechanism for liposome uptake is through opsonization with IgG and subsequent Fc-mediated phagocytosis or endocytosis [81].

Nabila et al. [82] observed that in liposomes containing certain lipids, including phosphatidylinositol, ganglioside G_{M1}, and sulfogalactosyl ceramide, complement-dependent phagocytosis of the liposomes was greatly suppressed. They suggested that suppression of this opsonization process could be a contributing factor in the promotion of increased circulation time of "stealth" liposomes and that complement opsonization probably plays a role *in vivo* in removing liposomes from the circulation.

Stabilization of the liposomal structure and therefore, decreased in the clearance rate can be obtained by inclusion of unesterified cholesterol that, by decreasing the fluidity of the lipid bilayer, renders it less easily penetrated by apoproteins and decreases

opsonization by plasma proteins [83]. For instance, it was found [84] that when cholesterol is incorporated in liposomes, the half-life was as long as 20 h.

The effect of surface charge on liposomal clearance has been investigated extensively [76, 77, 82, 83]. Senior et al. [83] found that negatively charged liposomes disappear from the circulation faster than neutral liposomes. Another study, however, indicated that this is not a general phenomenon and that some negatively charged lipids such as phosphatidylinositol and G_{M1} ganglioside can actually cause the retardation of liposomal clearance, due to suppression of the complement-dependent phagocytosis [82]. On the other hand, it was found that phosphatidylserine (PS) incorporated in liposomes accelerates clearance by the RES trapping via scavenger receptors on monophagocytes [77].

Abraham et al. [85] studied the pharmacokinetics of ^{3}H- triamcinolone acetonide-21-palmitate entrapped in liposomes with neutral, negative and positive surface in rabbits after single IV bolus injection. They found that positive liposomes had encountered a larger initial apparent volume of distribution than neutral or negatively charged liposomes. These results can be explained if it is considered that the rate of liposomal uptake by the RES is as follows: positive $\geq$ negative > neutral liposomes.

2.5.7.2 Disposition of liposomes following pulmonary administration

As mentioned above, one of the benefits of liposomes, as drug carriers is based on their ability to favorably alter the pharmacokinetic profile of the encapsulated species, and thus, provide selective and prolonged pharmacological effects at the site of administration. Administration of liposomes to the respiratory tract is attractive because of the

accessibility of the lung as a target organ, the compatibility of liposomes and lung surfactant components, and the need for sustained local therapy following inhalation. Due to this, numerous studies have explored the effect of liposomal encapsulation on the distribution and fate of compounds administered directly to the lung by either intratracheal instillation or inhalation.

One of the first studies was reported by Juliano et al. [86], comparing the effectiveness and distribution of free and encapsulated beta-cytosine arabinoside in MLV following intratracheal administration to rats. They found that free ARA-C (arabinoside C) was rapidly cleared from the lung ($t_{1/2}$ = 40 min) and entered the systemic circulation, while liposomal ARA-C displayed little redistribution to other tissues and persisted throughout the lung ($t_{1/2}$= 8h).

Farr et al. [87] investigated the pulmonary deposition and clearance of liposomes by labeling MLV and SUV composes of DPPC with ^{99}m-technetium. Deposition of nebulized vesicles was dependent on the droplet size of the aerosol produced, and not on the vesicle size. For both preparations, over 75% of the vesicles were retained in the lung after 6 hr, indicating that the mucociliary elevator was a major mechanism of clearance. Since the removal of particulate matter by the mucociliary elevator becomes progressively faster from the peripheral to central airways, the initial regional deposition of aerosol droplets must be considered an important factor in determining the rate of vesicle clearance. From a therapeutic point of view, alveolar deposition would be preferred, not only on the basis of providing local, but also prolonged activity due to the inability of the lung to clear vesicles as quickly from that site.

Woolfrey et al. [88] investigated the pulmonary absorption of liposome encapsulated 6-carboxyfluorescein (CF) following IT instillation to anaesthetized rats. The extent of CF absorption was influenced by the dose of lipid and the surface charge of the liposome. Increasing the lipid dose resulted in an increase in the rate of absorption, but decreased the extent of absorption. The authors suggested that the higher lipid dose accelerated removal of the liposomal CF from the lung, perhaps by specific pathways capable of removing particulate material. The absorption of CF from negatively charged vesicles was at least twice as fast as from neutral vesicles.

Investigations by Mihalko and co-workers [89], examined the effect of liposomal encapsulation on the pharmacokinetic fate of hydrophilic and lipophilic compounds following intratracheal instillation. They found that for hydrophilic compounds, the lung epithelium represented the major rate-limiting barrier for systemic absorption, not the rate of the drug release. In contrast, IT administration of lipophilic compounds as either free or encapsulated drug produced almost identical plasma levels suggesting that diffusion of the lipophilic compound through vesicles bilayers and lung was unrestricted.

A similar comparison of hydrophilic and lipophilic compounds was investigated by Meisner and colleagues [34] following endotracheal administration to rabbits. In this study, liposomal encapsulation of atropine base maintained drug concentrations in the lung approximately three-fold over 48-hr period while free atropine disappeared rapidly. After 48 hr, 21% of liposomal atropine was still associated with lung tissues as compared to 4% when instilled in solution form. They concluded that the amount of drug available to lung tissue from liposomes was controlled by the rate of release from the MLV.

Pulmonary delivery of liposomal encapsulated-glucocorticoids is rather scarce. One possible explanation for this is that glucocorticoids are highly lipophilic drugs which tend to escape very easily from liposomes when diluted in a larger volume, as has been demonstrated for hydrocortisone [90] and for triamcinolone acetonide [91]. In order to overcome this problem more hydrophilic derivatives of glucocorticoids have been used [23, 92, 93]. For example the encapsulation of triamcinolone acetonide-21-palmitate was 85%, compared to 5% for the parent drug [93]. Likewise, Brattsand et al. [23] demonstrated that budesonide 21-palmitate incorporated into liposomes, showed prolonged retention time (half-life = 6 hr) compared to budesonide, after intratracheal adminstration.

The practicability of aerosolizing liposomes for the inhalation and deposition of their content in the lung has been demonstrated [87, 91]. Traditionally, aqueous liposomes aerosols have been generated with nebulizers like the Collison and Hudson. However, it has been shown that the loss of an encapsulated marker is enhanced significantly by the air flow pressure, thus restricting the usefulness of nebulizers [94]. As an alternative to nebulization, emphasis has been put on the use of lyophilized liposomes delivered as dry powders with dry powder inhalers like the Spinhaler [95].

Biodistribution studies have demonstrated that liposomal encapsulation of compounds can localize and maintain drug levels in the lung for an extended period of time while decreasing the extent of systemic absorption. Pharmacologically, there is also evidence that liposomal drugs can produce selective and prolonged effects with decreased systemic toxicity. The pulmonary absorption of liposome-encapsulated solutes can be influenced by the physicochemical properties of the entrapped species, the site of

deposition in the lung as mentioned earlier, as well as, the lipid composition of the vesicles. There are 3 mechanisms that participate in the clearance of liposomes: mucociliary escalator, phospholipid exchange with the phospholipid pool and endocytosis by lung macrophages. Which mechanism takes place, depends also on the deposition in the lung. A better understanding of the factors that alter absorption and clearance is essential in designing a liposomal formulation that not only optimizes pharmacokinetics and pharmacological profiles, but also satisfies pharmaceutical requirements.

2.6 Synopsis of Literature Review

Asthma is perhaps the only treatable condition whose prevalence and severity are increasing. With the recognition of asthma as an inflammatory disease, glucocorticoids have become the drugs of choice for the treatment of this disease. Glucocorticoids exert their action directly by interaction of the receptor-glucocorticoid complex with certain portions of the DNA, or indirectly by inhibiting the activation of certain cytosolic factor such as AP-1 and NF-kB.

Inhalation therapy of glucocorticoids has the advantage of localizing the drug in the target organ, thereby reducing the dose, as well as, systemic exposure. Triamcinolone acetonide, beclomethasone dipropionate, budesonide, flunisolide and fluticasone propionate are the commercially available inhaled glucocorticoids for the treatment of asthma. They are delivered using metered dose inhalers (with and without spacer devices), nebulizers or dry powder inhalers. This relatively new generation of inhaled glucocorticoids are known to achieve higher local/systemic drug ratios than their oral counterparts, mainly because of their pharmacokinetic properties. They have higher

systemic clearance and lower oral bioavailability, which results in lower systemic drug concentration and therefore higher benefit/risk ratio. However, there are still concerns regarding side effects, especially the suppression of growth in children and osteoporosis, since these drugs are likely to be administered for long periods of time. Thus, identification of the factors that can improve pulmonary targeting is obvious.

Recently, it has been shown that not only clearance and oral bioavailability, but also drug release rate (slow release rate) and dose play an important role in pulmonary selectivity.

Liposomes (microscopic phospholipid vesicles) have been widely used due to their flexibility in controlling release rate. In the last two decades, it has been demonstrated that encapsulation of a drug into liposomes enhances its therapeutic efficacy, reduces its toxicity and prolongs its therapeutic effects. The concept of liposome administration to the respiratory tract is attractive because of the accessibility of the lung as a target organ, the compatibility of liposomes with lung surfactant components, and the need for sustained local therapy following inhalation. As a consequence, numerous studies have explored the effect of liposomal encapsulation on the distribution and fate of compounds administered directly to the lungs by either intratracheal (IT) instillation or inhalation.

Thus, the options for a further improvement of airway selectivity seem to be: 1) the design of glucocorticoids with extra-hepatic metabolism which lead to derivatives with even higher clearance and reduced systemic side effects and 2) the use of drug delivery systems such as liposomes or new drug entities which increase the pulmonary residence time. This thesis proposes the use of liposomes as a slow drug delivery system to improve pulmonary targeting.

CHAPTER 3
RESEARCH PROPOSAL

3.1 Objectives

One of the goals of the present study was to test whether the intratracheal (IT) administration of liposomes can improve pulmonary targeting. We also attempted to demonstrate that pulmonary targeting of glucocorticoids can be enhanced by optimization of dose and release rate. This goal was achieved by comparing pulmonary targeting of a liposomal formulation with different release rates and doses of encapsulated drug and relating them to pulmonary targeting of currently available inhaled glucocorticoids (powder formulations). Our ultimate goal was to identify some essential biopharmaceutical factors that are relevant for the development of aerosol dosage forms.

3.2 Specific aims

3.2.1 Aim # 1

To design a glucocorticoid-liposome dosage form suitable for pulmonary administration and assess pulmonary targeting of both, the developed liposomal glucocorticoid formulation and a control glucocorticoid in solution using and *ex vivo* animal model: assess size, encapsulation efficiency, lipid content, and *in vitro* stability in different biological fluids; and monitor glucocorticoid receptor occupancy in local organ

(lung) and in systemic organ (liver) upon intratracheal (IT) and intravenous (IV) administration.

3.2.1.1 Hypothesis

If the liposomal membrane serves as a rate limiting barrier for the release of the water-soluble TAP, then upon IT administration, a selective local (lung) receptor occupancy versus systemic (liver) receptor occupancy should be possible. In this way pulmonary targeting of encapsulated material will be more pronounced than pulmonary targeting of unencapsulated glucocorticoid (drug in solution).

3.2.1.2 Rationale.

We hypothesized that liposomes are a suitable dosage form due to their ability to act as drug carriers for pulmonary delivery and their flexibility in release rate. Phosphatidylcholine (PC), a neutral phospholipid and phosphatidylglycerol (PG), a negatively charged phospholipid, were chosen for the liposome preparation. This selection was based on the fact that these 2 compounds are biocompatible with lung surfactant that continuously lines the alveolar ducts. The lung surfactant pool is composed mainly of 60-70% of phosphatidylcholine and 5-10% of phosphatidylglycerol [96]. The use of negatively charged phospholipid not only increases the physical stability of the liposomes, but also favors the uptake by macrophages [67, 71, 88], ultimately, enhancing pulmonary targeting.

Among the great number of currently available glucocorticoids for the treatment of asthma, our group initially chose triamcinolone acetonide (TA) as a model drug. This drug

has the advantage of having a relatively short half-life, high receptor affinity, and low oral bioavailability, pharmacokinetic characteristics that favor pulmonary targeting. However, preliminary studies showed that while liposomes have a high loading capacity for TA under equilibrium conditions, it is rapidly released from the liposome matrix upon dilution. As the liposomes seemingly provide no barrier function for the lipophilic glucocorticoid TA, we believed that a solution to this problem was to encapsulate a water-soluble prodrug of TA. Thus, we selected the water-soluble triamcinolone acetonide phosphate (TAP), a prodrug of TA for the liposomal formulation. Since TAP is rapidly cleaved within minutes of administration (5-9 min) with complete conversion to its active compound TA [97], we expected almost immediate receptor interaction of TA once TAP is released from the liposomes.

The pharmacological effects of glucocorticoids are mediated through the interaction with receptors localized in the cytoplasm of the cells [25]. In addition, the degree of the pharmacological effects depends on the degree of receptor occupancy. Therefore, by simultaneously monitoring lung and liver receptor occupancy using an *ex vivo* animal model, it is possible to assess pulmonary and systemic side effects indirectly.

The use of liposomes has been suggested to provide sustained pulmonary release for various drugs including glucocorticoids such as beclomethasone dipropionate and dexamethasone [42, 98, 99]. However, such studies can not directly track pharmacodynamically relevant concentrations as generally both, encapsulated (pharmacologically inactive) and unencapsulated drug are being monitored. Because of these limitations, such an experimental design is not suitable for showing pulmonary targeting. The *ex vivo* receptor binding model employed in this study is able to quantify

the degree of pulmonary targeting by evaluating the difference between pulmonary and hepatic receptor occupancy. To determine the effect of the route of administration of pulmonary targeting we assessed lung and liver receptor occupancy after IT administration and compared it to lung and liver receptor occupancy after IV administration.

3.2.2 Aim # 2

Assess the pharmacokinetics and pharmacodynamics of the liposomal formulation after IT and IV administration and compare them to those after the intratracheal instillation of the drug in solution.

3.2.2.1 Hypothesis

The liposomal formulation will release the drug in a sustained release fashion, thus retarding the absorption of drug from the airways. The retention of drug in the lungs will result in a more prolonged mean residence time (change in the pharmacokinetics of the drug) as well as in sustained mean pulmonary effect (change in the pharmacodynamics) compared to those following instillation of the drug in solution.

3.2.2.2 Rationale

A number of relatively low lipophilic glucocorticoids (such as methyl prednisolone and triamcinolone acetonide) are rapidly absorbed from the airways because of their fast dissolution rate. Therefore, the encapsulation of these drugs into liposomes will control the release rate of these drugs , enhancing their retention in the respiratory airways. It has been stated that the overall effects (effect-time profile) depend not only on the

pharmacokinetics of the drug (concentration-time profile), but also on its intrinsic pharmacodynamics (effect-concentration profile) [100]. Thus, a change in the pharmacokinetics of the drug by slowing down its release rate will result in a favorably change in the drug pharmacodynamics (increase in pulmonary effects).

3.2 3 Aim # 3

To establish the relationship between liposome size and the release kinetics using 6-carboxyfluorescein (CF) as a marker: assess the efflux of CF in the presence of surfactant.

3.2.3.1 Hypothesis

If the interaction of liposomes with the surfactant pool present in the airways is the main mechanism of pulmonary clearance of liposomes, then the drug encapsulated in large vesicles will be retained longer than that encapsulated in small vesicles.

3.2.3.2 Rationale

It is known that the efflux of drug across the bilayers is the rate limiting step for the release of water soluble glucocorticoids [101]. In addition, it has been stated that the stability of liposomes (regarding leakage) depends primarily on liposome size and type [102]. Generally large multilamellar vesicles are more stable since only a portion of the phospholipid is exposed to attack, whereas small vesicles are the least stable because of the stress imposed by their curvature and the reduced number of lamellae in the membranes, thus increasing solute permeability. Although the *in vivo* mechanism(s) of

liposomes clearance may be completely different from the ones that govern the *in vitro* stability, assessing drug leakage from different sizes of liposomes under the same *in vitro* conditions may predict what happens *in vivo*.

CF has been widely used as a marker to determined the release kinetics from liposomes [88, 103, 104]. Like TAP, CF is a water soluble drug and thus, we expect that they partition in the same way in the liposome environment. A surfactant was included to mimic somehow the conditions that liposomes may encounter when delivered into the lungs.

These experiments, together with the *ex vivo* release studies performed in aim number 4, were intended to show that by changing the size of the liposomes and therefore the release rate, it is possible to optimize pulmonary targeting. This goal was achieved by assessing the release of 6-carboxyfluorescein from liposomes of different sizes in the presence of surfactant and by monitoring receptor occupancy after intratracheal instillation of TAP-liposomes of different sizes (see aim number 4).

3.2.4 Aim # 4

To determine the relationship between release rate, dose and pulmonary targeting: monitor lung and liver receptor occupancy upon intratracheal administration of glucocorticoid in solution and in liposomes with different release rates and different doses of encapsulated material.

3.2.4.1 Hypothesis

Pulmonary selectivity can be optimized by modulating drug release rate and dose.

3.2.5.2 Rationale

In attempts to increase localization of drug in the lung, drugs with higher clearance and lower oral bioavailability have been developed. However, little is known about the biopharmaceutical factors that affect pulmonary targeting. Pharmacokinetic-pharmacodynamic simulations done by our group [1] revealed that there are at least 2 biopharmaceutical factors that can affect pulmonary targeting: dose and release rate of the drug from the formulation (such as dissolution rate). Given a fixed dose, a value of release rate (K_{rel}) that is too small can mean that the minimum effect concentration is never reached either in the local organ (lung) or in the systemic organ (liver). When Krel is too high, toxic side-effects may result and pulmonary targeting is lost. The same rationale is applied for the dose. In this specific aim it was attempted to show experimentally that there is an optimal dose and release rate for which maximum pulmonary targeting can be achieved. For this purpose liposomes were used as a model dosage form due to their inherent capacity to control release rate as previously shown in the stability studies.

In order to compare the effect of release rate on pulmonary targeting, 3 different formulations were delivered intratrachealy to anesthetized male rats: triamcinolone acetonide phosphate (TAP) in solution (immediate release preparation), TAP in 200 nm liposomes (intermediate release preparation) and TAP in 800 nm liposomes (slow release preparation). Glucocorticoid receptor occupancy in the local organ (lung) was compared to glucocorticoid receptor occupancy in the systemic organ (liver). Pulmonary targeting

was calculated as the difference between the degree of lung receptor occupancy and the degree of liver receptor occupancy. To determine the effect of dose similar experiments were performed using escalating doses of TAP in 800 nm liposomes (TAP-lip 800 nm).

3.2.5 Aim # 5

Determine the pulmonary targeting of two currently available inhaled glucocorticoids: triamcinolone acetonide phosphate and fluticasone propionate using an *ex-vivo* animal model: monitor glucocorticoid receptor occupancy in local organ (lung) and in different systemic organs (liver, spleen and kidney) upon intratracheal (IT) administration of dry powders.

3.2.5.1 Hypothesis

If pulmonary selectivity depends on release rate then glucocorticoids of different dissolution rates will yield different pulmonary targeting

3.3.6.2 Rationale

Computer simulations [1] have shown that pulmonary selectivity can be modulated by optimizing drug release rate. In addition, the limited data available for currently available inhaled glucocorticoids suggest differences in their dissolution rates [105-108]. Based on these findings it is predicted that the pulmonary selectivity achieved by these drugs should be different. In this particular aim, kidney and spleen (organs with no metabolic activity) were included because of concerns on that the high intrinsic hepatic clearancc of fluticasone propionate may affect liver receptor occupancy.

CHAPTER 4
ANALYTICAL METHODS

4.1 Characterization of Liposomes

4.1.1 Size Measurement

The size (volume-weighted) and size uniformity of liposomes were determined using a submicron particle sizer (Nicomp, model 270). A drop of the liposome suspension was placed into a disposable culture tube (Fisher Scientific) and diluted with PBS (phosphate buffer saline) to obtain a photopulse rate of approximately 300 KHZ. The sample was monitored for 20 minutes or until the residual was zero and the fit error was smaller than two (the residual is a parameter which indicates the extent of a shift in the baseline of the autocorrelation function which is needed to produce the best fit for the Nicomp distribution analysis). Two different types of particle size distribution analyses were obtained: Gaussian and Nicomp. If the Chi Squared was less than 3.0, Gaussian analysis was displayed (Figure 4.1). If Chi Squared exceeded 3.0, the Nicomp distribution was displayed (Figure 4.2). Generally, 200 nm liposomes were best described by a Gaussian distribution, while 800 nm liposomes were best described by a Nicomp distribution.

4.1.2 Separation of Unencapsulated Material

Since the aqueous volume enclosed within the lipid membrane is usually only a small proportion of the total volume (about 5-10%) and triamcinolone acetonide phosphate is a water soluble compound, a step to remove unencapsulated material was needed. A size exclusion chromatography method [60] was used with some modifications.

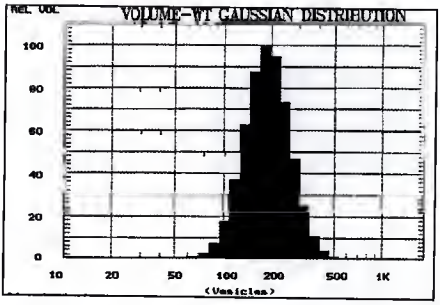

Figure 4.1 Volume weighted Gaussian distribution analysis for DSPC:DSPG (9:1) TAP-lip 200 nm

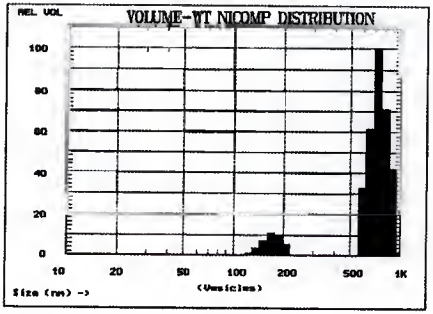

Figure 4.2 Volume weighted Nicomp distribution analysis for DSPC:DSPG (9:1) TAP-lip 800 nm.

4.1.2.1 Column preparation/processing of samples

Ten grams of Sephadex G-75 were allowed to swell in 120 ml of PBS for at least 10 hr at room temperature and stored at 4 °C. A PD-10 column (Pharmacia, Sweden) was plugged with a Whatman GF/B filter pad and the column was filled entirely with the hydrated gel using a Pasteur pipette with the tip removed. A second Whatman GF/B filter pad was attached on top of the hydrated gel, and 0.2 ml of liposome suspension (undiluted) was applied dropwise to the top of the gel bed. The first 3 ml of PBS (mobile phase) were added and the eluate discarded. After an additional 3 ml of PBS, the 4-6 ml eluate was collected. Liposomes were present in the 4-6 ml eluate. Preliminary studies using dyed liposomes prepared with the phospholipid rhodamine showed that 200 nm and 800 nm liposomes elute in the 4-6 ml fraction. Liposomes were stored at 4 ^{0}C under nitrogen.

4.1.3 Lipid Determination

The lipid contents of the liposomal preparations were determined by a slight modification of a colorimetric method [109].

4.1.3.1 Processing of samples

Duplicate samples of 0.010 ml of the liposome suspensions were evaporated using a Jouan concentrator for 1 hr followed by the addition of 3 ml of a mixture of 0.1M ferric chloride hexahydrate ($FeCl_3.6H_2O$) and 0.4M ammonium thiocyanate (NH_4SCN) in deionized water. The tubes were sonicated for 2 min, vortexed 15 seconds four times and incubated at room temperature for 1 hr. Subsequently, 4 ml of chloroform were added, the

tubes were closed and vortexed three times for 20 seconds. The samples were kept at room temperature for 2 hr or until a clear separation of the layers occurred. The aqueous (upper) layer was removed using a Pasteur pipette and the optical density (absorbance) of the chloroform layer was read at 488 nm using a UV/VIS spectrophotometer (Perkin Elmer, model λ-3A).

4.1.3.2 Calibration curve

For the calibration curve, 0.01-0.1 ml aliquots of a mixture of 90% PC and 10% PG (1 mg/ml total lipid in chloroform) were assayed in duplicate as described in section 4.1.3.

4.1.3.2.2 Inter- and intra-day variability

The intra-day and inter-day variability were determined for three control samples (20, 60 and 100 μg/ml) prepared as shown in 4.3.1. Replicate (n=5) analysis of control samples was performed on the same day and over a period of 7 days. Precision was expressed as the relative standard deviation obtained for the inter-day and intra-day analysis for each concentration. Accuracy was determined by comparing the resulting mean concentration with the theoretical one at each concentration. The correlation coefficients obtained for all calibration curves were always higher than 0.995. The coefficient of variation obtained for the slope was smaller than 12%. The results of the inter- and intra-day variability are shown in Table 4.1. Based on the results of the validation, the method was considered adequate for the purposes of this study.

Table 4.1 Intra-day and inter-day variability for DSPC:DSPG determination

Theoretical concentration (μg/ml)	Measured concentration (μg/ml) mean ± (SD)	Inter-day variability (%)	Intra-day variability (%)
20	21.2 (2.7)	13.64	12.9
60	52.9 (4.7)	4.96	8.8
100	101.7 (4.5)	1.55	4.9

4.1.4 Analysis of triamcinolone acetonide phosphate by HPLC

The applied reversed phase HPLC method for the quantification of encapsulated TAP has been previously described [110] and was used here with some modifications.

4.1.4.1 Instrumentation

The analysis of TAP was performed using a Perkin Elmer series 3B pump with a flow rate of 1.0 ml/min and a Nucleosil C_{18} (150 x 4.6 mm) column connected to a Perkin Elmer ISS 200 autoinjector. A water C_{18} guard pack column was used to protect the analytical column. The detector was a Milton Roy SM 4000 attached to a Hewlett Packard HP 3394A integrator. The eluent was monitored at 254 nm. The mobile phase consisted of a mixture of 35:65 v/v acetonitrile: sodium phosphate buffer 0.05 M (pH 2).

4.1.4.2 Treatment of samples

Liposomal TAP concentration was determined after gel filtration. Aliquots of 10 μl of the liposome dispersion were disrupted by adding 480 μl of a mixture of 80:20 methanol:PBS and 10 μl of methylprednisolone (100 μg/ml ethanol solution; internal standard). The mixture was vortexed for 1 min and 70 μl of the solution injected onto the

column. Quantitative analysis was achieved by comparison of peak heights with an internal standard using the fitted calibration curve.

Each determination was taken as the mean of two replicate injections. The calibration curve was prepared over the range of 75-800 μg/ml with duplicate samples. A typical triamcinolone acetonide phosphate and internal standard (methylprednisolone) chromatogram is shown in Figure 4.3.

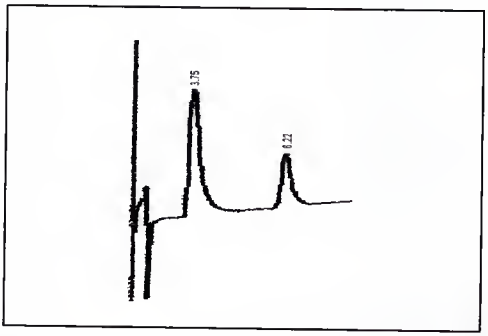

Figure 4.3 Representative chromatogram of TAP (RT 3.75 min) and internal standard (RT 6.22 min) in MeOH:PBS.

4.1.4.3 Intra-day and inter-day variability

The intra-day and inter-day variability were determined for three control samples (75, 30 and 600 μg/ml) prepared as shown in 4.1.4.1. The limit of quantification was determined as the lowest TAP concentration that produced an inter-day variability smaller than 20 %. The lower limit of quantification for this HPLC method was 30 μg/ml. The coefficient of variation obtained for the slope was 7.5% which reflects the precision of the

method. The correlation coefficient values were higher than 0.995. The results of the inter- and intra-day variability are shown in Table 4.2.

Table 4.2. Intra-day and inter-day variability for TAP reversed phase HPLC determination.

Theoretical concentration (μg/ml)	Measured concentration (μg/ml) mean ±(SD)	Inter-day variability (%)	Intra-day variability (%)
75	77.9 (6.7)	8.6	6.6
300	307.2 (10.9)	3.3	6.7
600	599.6 (12.7)	2.1	5.1

4.1.5 *In vitro* Stability at 37°C

The *in vitro* stability of freshly prepared TAP-lip was tested at 37°C using PBS, rat lung lavage fluid (see 4.1.6), and tissue culture medium RPMI-1640m or tissue culture medium with 10% fetal bovine serum. After gel filtration, an aliquot of the liposome preparation was diluted 5-fold (75-fold from the initial preparation) with the respective fluids. This resulted in a TAP concentration of 100 μg/ml, low enough to simulate sink conditions and high enough to be quantified by the HPLC system employed. After incubation at 37°C, aliquots of 200 μl (in duplicate) were removed at 10, 30 min, 1, 3, 6, 18, and 24 h and the samples were then passed through Sephadex G-50 dry minicolumns of 1 ml bed size [60] as is described in 4.1.5.1.

4.1.5.1 Column preparation/processing of samples

Ten grams of Sephadex G-50 were allowed to swell in 120 ml of PBS in a glass screw capped bottle for at least 4 hr at room temperature and stored at 4 °C. The plungers from 1-ml disposable plastic syringes were removed and plugged with a Whatman GF/B filter pad. The syringes were filled to the top with the hydrated gel using a Pasteur pipette with the tip removed, and centrifuged in a Dynac 11 centrifuge (Becton, Dickinson and Company) at 2000 rpm for 5 min to remove excess of solution. Duplicated aliquots of 0.2 ml of the liposome suspension were applied dropwise to the top of the gel bed and spun down at 2000 rpm for 2 min. Aliquots of 10 µl eluates were analyzed by HPLC (see 4.1.4).

4.1.6 Method for Recovering Lung Lavage Fluids

Rats were anesthetized and lung lavage fluid was obtained after tracheal cannulation with a 21 gauge polyethylene catheter. Approximately 5 ml of sterile isotonic saline were slowly injected to fill the lungs. The fluid was withdrawn by gentle aspiration, and spun at 2000 rpm for ten minutes at 4°C. The cell free supernatant was aspirated and used on the same day to assess the *in vitro* stability of liposomes.

4.2 Receptor Binding Assay

Receptor binding was performed as previously described [56]. Immediately after decapitation, the lung, without trachea, and a lobe of the liver were resected and placed on ice. The weighed tissue was added to 10 ml volumes of ice-cooled incubation buffer (10 mM Tris/HCl, 10 mM sodium molybdate, 2 mM 1,4-dithiothreitol) and homogenized in a Virtis 45

homogenizer at 40% of full speed, for three periods of 5 seconds each with a 30 second cooling period between each step. One tenth volume of 5% activated charcoal (prepared in incubation buffer) was added to the homogenate and mixed. After 5 minutes, the suspension was centrifuged at 4°C at 50,000g for 10 min. in a Beckman centrifuge equipped with a JA-21 rotor (Beckman instruments, Palo Alto, CA) to obtain a clear supernatant. Aliquots of the supernatant (150 µl) were transferred into microcentrifuge tubes that contained 25 µl of ^{3}H-triamcinolone acetonide in incubation buffer (final concentration: 0.25, 0.5, 2, 4, 10 and, 30 nM) and 25 µl of incubation buffer or 25 µl of unlabeled TA (3 mM) to determine total and non-specific binding respectively. Aliquots of 150 µl of the resultant cytosol preparations were transferred into microcentrifuge tubes which contained 25 µl of ^{3}H-triamcinolone acetonide in incubation buffer (final concentration: 0.25, 0.5, 2, 4, 10 and 30 nM) and 25 µl of incubation buffer to determine the amount of total radioactivity. In some cases only high concentration (30 nM) of labeled material was used (see chapters 7 and 8). After a 16-24 hour incubation period at 4°C, the unbound glucocorticoid was removed by addition of a 2% suspension of activated charcoal in buffer (200 µl). The mixture was incubated for 5 min on ice and then centrifuged at 10,000 rpm for 5 min in a micro-centrifuge (Fisher model 235A). The radioactivity (dpm) in 300 µl of supernatant was determined using a liquid scintillation counter (Beckman model LS 5000 TD, Palo Alto, CA). All determinations were performed in triplicate.

Estimates for total bound ^{3}H-TA (TA_T), nonspecifically bound ^{3}H-TA (TA_{NS}) and free ^{3}H-TA (TA_F) were used to determine the number of available binding sites (B_{max}) and the equilibrium binding constant (K_d). The data were fitted to equation 4.1 using the non-linear curve-fitting program MINSQ (Micromath, Salt Lake City, UT).

$$TA_T = B_{max} * TA_F / (TA_F \pm K_d) \pm TA_{NS} \qquad \text{(eq. 4.1)}$$

In some cases, B_{max} could not be determined by the above method, as the high in vivo receptor occupancy did not allow a reliable estimate of B_{max} and Kd. In this case, B_{max} was estimated by a second method and expressed as the difference of TA_T values and TA_{NS} observed for incubation with 30 nM ^{3}H-TA (highest tracer concentration). This was justifiable as the comparison of calculated B_{max} values from both methods resulted in similar values for B_{max} (R^2=0.985, n=45; see Figure 4.3).

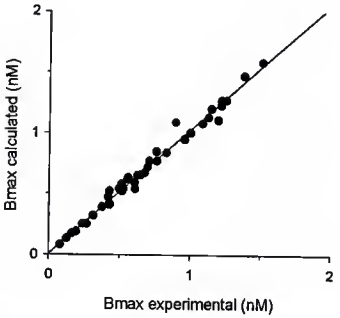

Figure 4.3 Correlation between B_{max} calculated values (using equation 4.1), and B_{max} experimental (obtained from the difference of TA_T and TA_{NS} observed values). R^2=0.985, n=45.

4.3 Reverse HPLC and Radioimmunoassay (HPLC/RIA) Determination of Triamcinolone Acetonide

4.3.1 Extraction Procedure

Plasma and lung samples were thawed at room temperature for one hour and extracted twice with 2 ml of ethyl acetate as described in detail somewhere else [111]. The organic solvent was evaporated under vacuum in a speed vacuum concentrator (Jouane). The residue was reconstituted in 150 µl of 50:50 mobile phase (acetonitrile:water) and the resulting suspension centrifuged for five minutes. The supernatant was used directly for the HPLC separation.

4.3.2 Reverse Phase HPLC Procedure

The analysis of TA was performed using a Costametric 111G pump (LDC/Milton Roy) to deliver solvent at 0.9 ml/min to a Sperisorb ODSII (150 x 4.6 mm) C18 column connected to a Perkin Elmer 1SS 200 autoinjector and to a programmable Gilson model 203 fraction collector. The spectromonitor D (LDC) variable UV detector was attached to a Hewlett Packard HP 3394A integrator. The eluent was monitored at 254 nm. The mobile phase consisted of a mixture of acetonitrile: water (50:50 v/v). Retention times of hydrocortisone and triamcinolone acetonide were verified before every experiment. After extensive washing, plasma or lung extracts (200 µl) were injected and the TA fractions collected. Organic solvent containing TA (HPLC fractions collected) was removed under vacuum. The residual material was extracted twice with ethyl acetate (1 ml), centrifuged and the solvent evaporated until dryness. The resulting residues were reconstituted in 300

µl of incubation buffer containing 0.001% Triton X-100 and centrifuged. The portions of the supernatant were used for RIA procedure.

4.3.3 RIA Procedure

The dried residue was dissolved in 300 µl of 0.01 M PBS containing Triton X-100. Lung or plasma samples (50 µl), 200 µl of assay buffer, 50 µl of antiserum working solution (1:1500 antibody), and 50 µl of tracer solution (^{3}H-TA, 3000 CPM) were placed in microcentrifuge tubes and incubated for 24 h at 4° C. After incubation, 100 µl of 1 % activated charcoal was added to the samples. The samples were incubated for 5 min and centrifuged at 10,000 rpm. Aliquots of the supernatant (600 µl) were mixed with 4 ml of scintillation fluid and counted in a scintillation counter.

Calibration curves of the bound radioactivity (B) versus competitor concentration (C) were fitter to the equation shown below using a non-linear curve fitting procedure (Scientist, Micromath, Salt Lake City, Utah).

$$B = T - T^{*}C^{N}/(CN + IC_{50}^{N}) + NS$$

Estimates if NS (non specific binding), N (Hill slope factor), IC_{50} (concentration to decrease specific tracer binding by 50%), and total specific binding (T) were used to transform CPM of the unknowns into the corresponding concentrations.

The limit of quantification was 0.15 ng/ml (accuracy 85-115% and precision 15 %) and the limit of detection was 0.1 ng/ml. The inter-batch variability for three batches of quality controls samples was in average 13, 11, and 11 % for 0.35, 0.75, and 1.5 ng/ml. Accuracy was between 99 and 118 %. Intra-day variability for quality control samples was 5.2, 4.7, and 4.4 % for 0.35, 0.75, and 1.5 ng/ml, respectively.

CHAPTER 5
PULMONARY TARGETING OF LIPOSOMAL TRIAMCINOLONE ACETONIDE PHOSPHATE

5.1 Introduction

Glucocorticoids are beneficial in treating various pulmonary diseases, including asthma, sarcoidosis, and other conditions associated with alveolitis. Although systemic glucocorticoid therapy is effective in such conditions, prolonged administration carries the risk of toxicity and side effects [112]. In attempts at reducing systemic side effects, several clinically efficacious glucocorticoids, including triamcinolone acetonide (TA) are employed for delivery as aerosols.

We have been interested in optimizing pulmonary targeting of glucocorticoids for inhalation therapy. In a recent study, we showed that lung specificity is achieved when glucocorticoid suspensions are administered intratracheally. In contrast, lung targeting is not observed when a glucocorticoid solution is administered intratracheally, presumably because of the fast absorption of the lipophilic steroid [56]. This suggests that pulmonary targeting depends on slow release from the delivery form which results in a prolonged pulmonary residence time.

The use of liposomes has been suggested to provide sustained pulmonary release for various drugs including glucocorticoids such as beclomethasone dipropionate, and dexamethasone [42, 98, 99, 113]. However, we have found that while liposomes have a high loading capacity for lipophilic glucocorticoids such as TA under equilibrium

conditions, TA is rapidly released under non-equilibrium conditions from the liposome matrix upon dilution or administration [91]. In retrospect, this is predictable, given the observations of Schanker & co-workers [49] that lipophilic glucocorticoids cross membranes practically unhindered. Our findings question the benefits of achieving sustained pulmonary release from such preparations.

As liposomes seemingly provide no barrier function for such glucocorticoids we hypothesized that by encapsulating water-soluble derivatives of TA, rather than the lipophilic parent compounds we could overcome this problem. If the liposomal membrane serves as a rate-limiting barrier for the release of a water-soluble TA derivative, then slow drug release and consequent improvement in pulmonary targeting might be possible.

We therefore selected the water-soluble salt triamcinolone acetonide phosphate (a prodrug of TA) for developing a formulation which captured the negatively charged TAP within liposomes. This formulation was delivered either by intratracheal or intravenous injection to rats and compared with intratracheally administered TAP-sol. Since pharmacodynamic effects of glucocorticoids are receptor mediated, tracking receptor occupancy in lung and liver using the above mentioned animal model allowed for indirect assessment of pulmonary and systemic effects.

5.2 Materials and Methods

5.2.1 Materials

Analytical grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO.); (1,2,4-^{3}H) triamcinolone acetonide (45 Ci/mmol) was purchased from New-England Nuclear (Boston, MA). Lipids were obtained from Avanti Polar Lipids (Alabaster, AL.)

5.2.2 Drug Solutions

Triamcinolone acetonide phosphate (TAP) (provided as a gift from Dr. K. Reininger, Bristol Myers Squibb, Regensburg, Germany) was dissolved in buffered saline to a concentration of 15 mg/ml. Before dosing, the respective stock solutions were diluted with buffered isotonic saline to 0.2 mg/ml of TAP.

5.2.3 Animals

All animal procedures were approved by the Animal Care Committee of the University of Florida, an AAALAC approved facility. Specific-pathogen-free, non-adrenalectomized male F-344 rats, weighing approximately 250 g were housed in a 12 hr light/dark cycle, in a constant temperature environment. Animals were allowed free access to water and rat chow, but were food-fasted overnight prior to each experiment.

5.2.4 Liposome preparation

Liposomes composed of 1,2-distearoyl-sn-glycero-3-phosphocholine (89.4 mg) and 1,2-distearoyl-sn-glycero-3-{phospho-rac-(1-glycerol)} (10.6 mg) in a 9:1 molar ratio respectively, were dissolved in chloroform. The chloroform was removed by rotary evaporation at 63°C to obtain a dry lipid film. After adding one ml of 100 mg/ml TAP solution in isotonic buffer saline (PBS, Cellgro™, pH 7.5) the lipids were dispersed by shaking at 63°C for 2hr. This crude liposome formulation underwent 10 cycles of freezing (in dry ice and methanol) and thawing at 63°C and was then extruded 30 times using a Liposofast® extruder (Avestin Co. Ottawa, Ontario, Canada) through 0.2 mm polycarbonate filters. Liposomes were sized using a submicron particle sizer (Nicomp model 270) to ensure size uniformity of the formulations, and stored under nitrogen at 4°C.

On the day of the experiment, 200 µl of liposomal dispersion were passed through 10 ml Sephadex G-75 in a PD-10 column (Pharmacia, Sweden) with a PBS mobile phase. Liposomes were present in the first 4 to 6 ml of eluate (void volume). TAP concentrations were monitored by HPLC (section 5.2.5) For animal experiments, liposomes were diluted to a final concentration of TAP of 0.2 mg/ml. Isotonicity of liposomes was confirmed prior to use with a vapor pressure osmometer M 5500 (Wescor, Inc. Logan, UT). Lipid content was determined as previously described [109].

5.2.5 HPLC Method

Liposomal TAP concentration after gel filtration was determined in a 10 µl aliquot of the liposome dispersion by adding 480 µl of a mixture of 80:20 methanol:PBS and 10 µl of methylprednisolone (100 µg/ml ethanol solution) as internal standard. The mixture was vortexed for 1 min and 70 µl of the solution was injected onto a Nucleosil C_{18} (150 x 4.6 mm) column, using a mixture of acetonitrile:0.05 M sodium phosphate buffer (pH 2; 35:65 v/v), as mobile phase at a flow rate of 1 ml/min. UV detection was performed at 254 nm. The lower limit of detection was 30 µg/ml.

5.2.6 Method for recovering lung lavage fluids.

Rats were anesthetized and lung lavage fluid was obtained after tracheal cannulation with a 21 gauge polyethylene catheter. Approximately 5 ml of sterile isotonic saline were slowly injected to fill the lungs. The fluid was withdrawn by gentle aspiration, and spun at 2000 rpm for ten minutes at 4°C. The cell free supernatant was aspirated and used on the same day to assess the in vitro stability of liposomes.

5.2.7 *In vitro* Stability

The in vitro stability of freshly prepared TAP-lip was tested at 37°C using PBS, rat lung lavage fluid, tissue culture medium RPMI-1640m or tissue culture medium with 10% fetal bovine serum. After gel filtration, an aliquot of the liposome preparation was diluted 5-fold (75-fold from the initial preparation) with the respective fluids (TAP final concentration of 100 µg/ml, a concentration that simulates sink conditions and is high enough to be quantified by the HPLC system employed).

After incubation at 37°C,aliquots of 200 μl (in duplicate) were removed at 10, 30 min, 1, 3, 6, 18, and 24 h and the samples were then passed through Sephadex G-50 dry minicolumns of 1 ml bed size [60]. Aliquots of 10 μl eluates were analyzed by HPLC.

5.2.8 Administration of Drugs

The animals were anesthetized via intraperitoneal injection and the corresponding TAP-lip or TAP-sol (160 μg/kg of TAP) administered [56]. Animals (one animal per time point) were decapitated at 1, 2, 5, 6, 12, or 18 hours after IT or IV administration of Tap-lip. The lungs and livers were immediately processed for receptor binding studies. A total of 3 (12 and 18 hr) to 6 (0-6 hr) independent experiments were performed for a given time point after IT administration of TAP-lip. For the IT administration of TAP-sol and the IV administration of TAP-lip 4 and 2 animals were used per time point, respectively.

Experiments were performed on different days for every form (IV or IT) of administration and for each type of preparation, e.g. TAP-sol or TAP-lip. A sham animal (receiving either IV or IT buffered saline) was always included on the day of experiment. Each animal represented a single time point with paired data for both liver and lung.

5.2.9 Receptor Binding Assays

Receptor binding assays were performed as previously described [56] with modifications. Immediately after decapitation, the lung, without trachea, and a lobe of the liver were resected and placed on ice.

The weighed tissue was added to 10 ml volumes of ice-cooled incubation buffer (10mM Tris/HCL, 10 mM sodium molybdate, 2mM 1,4-dithiothreitol) and homogenized

in a Virtis 45 homogenizer at 40% of full speed, for three periods of 5 seconds each with a 30 sec cooling period between each step. One tenth volume of 5% activated charcoal (prepared in incubation buffer) was added to the homogenate and mixed. After 5 minutes, the suspension was centrifuged at 4°C at 50,000g for 10 min. in a Beckman centrifuge equipped with a JA-21 rotor (Beckman instruments, Palo Alto, CA) to obtain a clear supernatant.

Aliquots of the supernatant (150µl) were transferred into microcentrifuge tubes that contained 25 ml of ^{3}H-triamcinolone acetonide in incubation buffer (final concentration: 0.25, 0.5, 2, 4, 10 and, 30 nM) and 25 ml of incubation buffer or 25 ml of unlabeled TA (24mM) to determine total and non-specific binding respectively. Aliquots of 150 ml of the resultant cytosol preparations were transferred into microcentrifuge tubes which contained 25 ml of ^{3}H-triamcinolone acetonide in incubation buffer (final concentration: 0.25, 0.5, 2, 4, 10 and 30 nM) and 25 ml of incubation buffer to determine the amount of total radioactivity.

After a 16-24 hour incubation period at 4°C, the unbound glucocorticoid was removed by addition of a 2% suspension of activated charcoal in buffer (200 ml). The mixture was incubated for 5 min on ice and then centrifuged at 10,000 rpm for 5 min in a micro-centrifuge (Fisher model 235A). The radioactivity (dpm) in 300 ml of supernatant was determined using a liquid scintillation counter (Beckman model LS 5000 TD, Palo Alto, CA). All determinations were performed in triplicate.

Estimates for total bound ^{3}H-TA (TA_T), nonspecifically bound ^{3}H-TA (TA_{NS}) and free ^{3}H-TA (TA_F) were used to determine the number of available binding sites (B_{max}) and

the equilibrium binding constant (K_d). The data were fitted to equation 1 using the non-linear curve-fitting program MINSQ (Micromath, Salt Lake City, UT).

$$TA_T = B_{max} * TA_F / (TA_F + K_d) + TA_{NS} \qquad \text{(eq. 5.1)}$$

In instances where the high in vivo receptor occupancy did not allow for reliable B_{max} determination by the above method, B_{max} was estimated as the difference of TA_T values and TA_{NS} observed for incubation with 30 nM ^{3}H-TA (highest tracer concentration). This was justifiable as the comparison of calculated B_{max} values from both methods resulted in similar values for B_{max} (R^2=0.985, n=45).

Pulmonary and hepatic B_{max} values were converted into % of control (average values for sham rats). K_d values were necessary only to calculate B_{max}, and are not given in the result section.

For a given form of administration, differences between the pulmonary and hepatic receptor occupancies were tested by paired student T test. Data analyzed represented the pool of paired (hepatic and pulmonary) receptor occupancies for individual single time points (not AUC's) of all animals included in a given experimental sub-set. Statistical significance was assumed for $p<0.05$.

For assessing differential receptor occupancy between lung and liver, the cumulative change from baseline (AUC) was calculated for the 6 and 18 hour investigation period by the trapezoidal rule from percent occupied receptor (E_x)-time profiles. The pulmonary targeting factor (T) was defined as

$$T=AUC_{Lung}/AUC_{Liver} \qquad \text{(eq. 5.2)}$$

A targeting factor of greater than 1 would indicate preferential lung targeting. The area under the first moment curve ($AUMC_\infty$) was calculated by the trapezoidal rule from

E_X*t_X versus t_X-pairs. Effects after the last measurement points were extrapolated for TAP-sol and data of reference 2 assuming a linear decline of the effect over time at late time points [100]. These estimates were also used to derive AUC_∞. The mean pulmonary effect times (MET) were calculated consequently from AUC_∞ and $AUMC_\infty$ (MET= $AUMC_\infty/AUC_\infty$).

5.3 Results

5.3.1 Characterization of Lip TAP

The volume weighted mean particle diameter of the liposome obtained using a Gaussian distribution analysis was 207 ± 16 nm (see Figure 4.1, chapter 4). A 40% average loss of lipid resulted after extrusion and gel filtration of liposomes. The encapsulation efficiency varied from 7 to 8.5% of the total amount of TAP, corresponding to a molar drug/lipid ratio of 1:7.

5.3.2 *In vitro* Stability in Biological Fluids

Results of the liposome stability studies are shown in Figure 5.1. The TAP-lip preparations retained greater than 75-80% of the entrapped drug through 24 hours when incubated in various fluids.

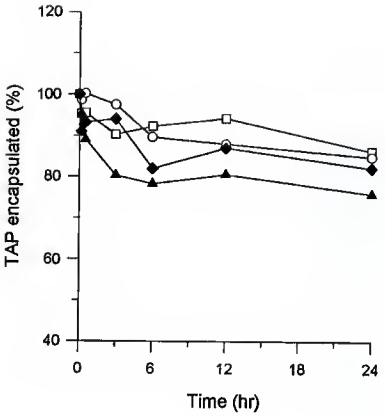

Figure 5.1. *In vitro* stability of DSPC:DSP TAP liposomes at 37°C under sink conditions in PBS (◆), culture medium (□), lung lavage fluid (○), and 90% culture medium plus 10% fetal bovine serum (▲). The percentage of TAP remaining encapsulated in liposomes over time of incubation is shown.

5.3.3 Receptor Binding Studies

To exclude potential assay artifacts (e.g. ex-vivo release of glucocorticoids from intact liposomes present in resected tissues), experiments were performed in which cytosol from untreated animals was spiked with TAP-lip (TAP 200 μg/ml) and consequently processed as described in Materials and Methods. There was a negligible difference between the % of free receptors of cytosol spiked with liposomes (85% ± 10%, n=4) and control cytosol not spiked with TAP-lip (B_{max} = 100%).

There were statistically significant differences in glucocorticoid receptor occupancy between lung and liver after intratracheal administration of TAP-lip for the 18

hr period. Receptor occupancy was more pronounced for lung than for liver (Fig. 5.2b), reflected also in an AUC based lung targeting factor of 1.6 (Table 5.1). In contrast, there was no difference in these parameters when equivalent doses of TAP were administered IT as a solution, with the lung and liver curves being superimposable (Fig. 5.2a, Table 5.1).

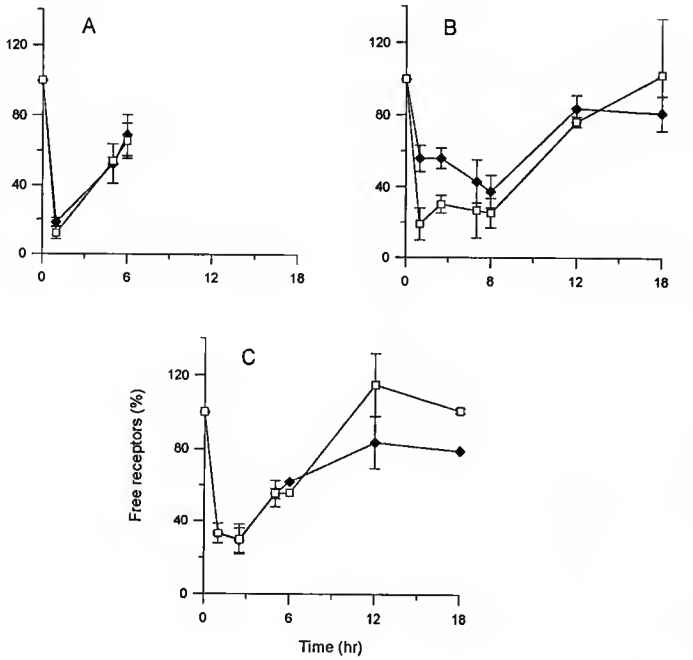

Figure 5.2. Lung (□) and liver (◆) glucocorticoid receptor occupancy profiles after administration of 100 μg/kg of TAP: A) intratracheal instillation of TAP-sol; B) intratracheal instillation of TAP-lip; C) intravenously administered TAP-lip. Error bars represent mean ± SD

Table 5.1 Cumulative receptor occupancy (AUC), lung targeting factor and mean pulmonary effect times (MET) after administration of TAP liposomes (TAP-lip) and TAP solution (TAP-sol).

	TAP-lip it adm		TAP-sol it adm	TAP-lip iv adm	
	AUC (%*hr)				
	0-6hr	0-18hr	0-6hr	0-6hr	0-18hr
LUNG	450	770	350	490	370
LIVER	280	620	340	500	600
Targeting factor (AUC_{lung}/AUC_{liver})	1.6	1.2	1.0	1.0	0.6
Mean Pulmonary Effect Time (hr)	5.7		3.0	3.8	

After IV administration of TAP-lip, no significant differences were observed in receptor occupancy profiles of lung and liver (Fig. 5.2c, Table 1) for at least the first 6 hours.

The lung receptor occupancy profiles between the various TAP drug preparations and methods of administration are compared in Figure 5.3 together with previously reported data on triamcinolone acetonide solutions (TA-sol) [100]. As is shown in this Figure, lung receptor occupancy was significantly sustained after IT administration of TAP-lip relative to IT and IV of TAP-sol and TAP-lip respectively, and to IT or IV administered TA-sol. The distinct sustained receptor occupancy characteristics of TAP-Lip can be seen also from the estimates of the mean effect times (Table 5.1). These MET

values were 1.5-1.9 times longer than that of TAP-lip administered IV and TAP-sol administered IT (Table 5.1), respectively.

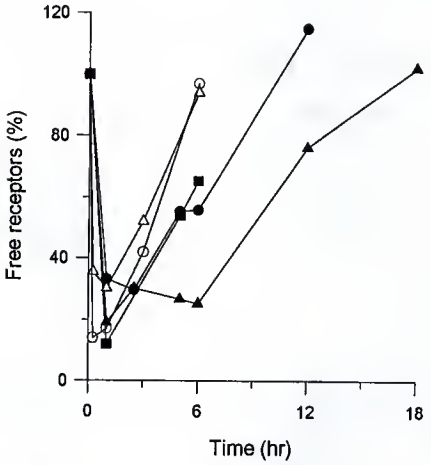

Figure 5.3. Lung glucocorticoid receptor occupancy profiles after administration of 160 μg/kg of TAP: Intratracheal TAP-sol (■), intratracheal TAP-lip (▲), intravenous TAP-lip (●). Comparison is made to intratracheal TA-sol (Δ) and intravenous TA-sol (○) profiles from ref. [56].

5.4 Discussion

TA is a clinically established, potent and inhalable synthetic glucocorticoid which binds to the glucocorticoid receptors. In contrast to TA, TAP (a prodrug of TA) is highly water-soluble (sol in water > than 50 mg/ml), and although the encapsulation efficiency was substantially less than that of TA [91], we found TAP-lip (unlike TA-lip) to be more stable, even when incubated in buffers or biological fluids at 37°C (Figure 5.1).

In the systemic circulation, TAP is cleaved rapidly to the pharmacologically active triamcinolone acetonide [97]. Our results in rats are in agreement with prior observations, as the receptor occupancy profiles after administration of the TAP-sol are very similar to what we have previously shown for TA when administered in the same fashion (Figure 5.3), suggesting a fast activation of TAP. In addition, the distinct pulmonary targeting of liposomal TAP suggests that this activation has to occur in the lung.

Incorporation of drugs into liposomes changes their pharmacokinetic behavior after pulmonary instillation [99], resulting in longer mean residence time in the lung and the potential for sustained pulmonary effects. Based on our experiments, intratracheal administration of TAP-lip resulted in a pronounced and sustained occupancy of pulmonary receptors when compared to pulmonary delivery of TAP-sol or intravenously administered TAP-lip (Figure 5.3, Table 5.1). This suggests that the sustained receptor occupancy is linked to the pulmonary administration of the liposomal preparation, while the use of TAP-sol or the intravenous administration of TAP-lip do not result in the sustained occupation of pulmonary receptors (Figure 5.3). These results are consistent with the *in vitro* stability of the TAP liposomes in biological fluids (Figure 5.1) and prolonged pulmonary release of TAP from the liposomes.

In pharmacokinetic-pharmacodynamic (PK-PD) simulations we have shown that the MET depends on the release rate of the drug delivery system in such a way that the slower the release, the larger the MET (unpublished observations). On the basis of this relationship, the longer MET value found for TAP-lip argues also for a prolonged release of this formulation.

Besides providing a sustained receptor occupancy, the main goal of this study was to test whether liposomes can improve pulmonary targeting after topical delivery. Previous studies [56] using TA suspensions and solutions suggested that pulmonary targeting, as indicated by a ratio of the cumulative pulmonary and hepatic receptor occupancy, is achieved only if the drug is absorbed slowly from the lung; e.g. due to a slow dissolution rate of the instilled particles. This study shows that TAP-lip, with presumably prolonged release characteristics, but not TAP-sol confers pulmonary targeting. The resulting targeting factor of 1.6 was significantly larger than estimates for TAP-sol (1.0) or IV liposomes.

Although achieving a pronounced targeting for tissues with high organ blood flow, such as the airways is theoretically difficult [114], our results show that liposomes increase the therapeutic availability of drugs in target tissue (presumably due to prolonged release) therefore increasing tissue targeting.

Our study shows that triamcinolone acetonide phosphate can be incorporated in a stable liposomal formulation which appears suitable for pulmonary delivery. With intratracheal delivery, TAP-lip provides sustained receptor occupancy properties and improved pulmonary targeting superior to TAP-sol alone. Whether this formulation will

ultimately result in superior clinical activity in pathologic states in humans cannot be ascertained, but deserves further study.

CHAPTER 6
PHARMACOKINETICS AND PHARMACODYNAMICS OF TRIAMCINOLONE ACETONIDE PHOSPHATE IN LIPOSOMES AND IN SOLUTION AFTER INTRATRACHEAL AND INTRAVENOUS ADMINISTRATON

6.1 Introduction

Because asthma is considered an inflammatory disease [28], glucocorticoids are the drugs of choice for the treatment of this disease. Generally, glucocorticoids are administered locally as aerosols in an attempt to reduce systemic side effects. However, due to its rapid absorption into the systemic circulation, frequent administration of these drugs is needed leading to potential side effects [115, 116].

This prompted the development of slow release preparations (such as liposomes) for pulmonary delivery (for recent review see [42] to control the absorption of drugs from the respiratory airways. Liposomes have been suggested to provide a prolonged release reservoir and facilitate the intracellular targeting of drugs [42]. To date, only few reports have addressed the use of liposome-encapsulated glucocorticoids for pulmonary delivery [23, 99]. Furthermore, little is known about the pharmacokinetics and pharmacodynamics of liposomal encapsulated glucocorticoids for pulmonary delivery.

We have previously shown (chapter 5) that the intratracheal (IT) administration of TAP in liposomes resulted in a more pronounced lung receptor occupancy compared to TAP in solution or TAP administered intravenously (IV). In the present study, we correlate the pharmacokinetics of TA after IT administration of TAP liposomes with its

pulmonary and systemic effects (receptor occupancy) and compare these with those of TAP in solution administered IT and after IV administration of TAP liposomes.

6.2 Methods

The methods for liposome preparation and characterization, drug administration and receptor binding were reported previously in chapter 5.

Briefly, triamcinolone acetonide liposomes composed of phosphatidylcholine, phosphatidylglycerol and triamcinolone acetonide phosphate (TAP) were prepared by dispersion and extruded through 200 nm polycarbonate filters. Encapsulation efficiency and *in vitro* stability at 37 °C were assessed by size exclusion chromatography (refer to section 3.1.2 and 3.1.5). TAP liposomes (TAP-lip) or TAP in solution (TAP-sol) were delivered to male rats either by IT instillation or IV administration (chapter 5). Glucocorticoid receptor occupancy was monitored over time in the lung and liver using an *ex vivo* receptor binding assay as a pharmacodynamic measure of glucocorticoid action (section 3.2).

6.2.1 Quantification of Triamcinolone Acetonide (TA) in Plasma and Lung Samples

Following drug administration (100 μg/kg of TAP in either liposomes or solution), animals were decapitated at 1, 2, 5, 6, 12, or 18 hours (chapter 5). Blood samples were collected in glass tubes containing heparin and centrifuged at 2,000 rpm for 20 minutes. The plasma was separated and stored at -20 °C until analyzed. Lungs were resected, homogenized as described previously (chapter 5) and samples were frozen (- 20 °C) until analyzed.

The plasma and lung concentrations of triamcinolone acetonide were determined by radioimmunoassay after reversed-phase HPLC separation from metabolites and endogenous glucocorticoids as described in section 3.3. Briefly, plasma and lung samples (1 ml) were thawed for 1 h at room temperature and extracted twice with 2 ml of ethyl acetate. The organic phase was evaporated under vacuum, reconstituted in 0.15 ml of mobile phase and centrifuged for 5 minutes at 10,000 rpm. Aliquots (200 µl) of the supernatant were injected onto a Spherisorb ODS2 (150x4.6 mm, 5µm) column, using acetonitrile/water (50:50) as a mobile phase at a flow rate of 0.9 ml/min. The HPLC fractions containing TA were collected, evaporated, and extracted twice with ethyl acetate. The organic solvent was evaporated under vacuum, reconstituted in incubation buffer and analyzed by radioimmunoassay. The limit of quantification was 0.15 ng/ml with an average coefficient of variation of 11% in a concentration range of 0.35 to 1.5 ng/ml.

6.3 Data Analysis

6.3.1 Pharmacokinetic Analysis

Plasma and lung TA average concentrations over time were subjected to standard compartmental and non-compartmental pharmacokinetic analysis.

6.3.1.1 Compartmental analysis

6.3.1.1.1 Lung concentrations

Average TA lung concentrations (CL_{TAL}) versus-time profiles upon IV and IT administration of TAP-lip were fitted to a two-compartment body model using the least-squares nonlinear regression method (SCIENTIST, Micromath, Salt Lake City, UT). Average concentration of TA were fitted to the following equation:

$$C_{TAL} = A\, e^{-\alpha t} + B\, e^{-\beta t} \qquad \text{(eq. 6.1)}$$

where C_{TAL} is the total TA concentration, α, and β are first order rate constants, while A and B represent hybrid constants. In the case of TAP-sol administered IT the equation used to fit the lung data was:

$$C_{TAL} = A\, e^{-k_e t} \qquad \text{(eq. 6.2)}$$

where k_e is the first order elimination constant and A is the concentration at time zero.

The area under the lung concentration-time curve ($AUC_{0\rightarrow t}$) after intravenous and intratracheal administration was calculated by the trapezoidal rule. Interpolation to infinity was done by dividing the last C_{TAL} measured by the first-order rate constant of the terminal phase. The total area under the curve was estimated by the summation of these two components.

$$AUC_{0\rightarrow\infty} = (AUC_{0\rightarrow t}) + (AUC_{t\rightarrow\infty}) \qquad \text{(eq. 6.3)}$$

The elimination half-life was calculated from $0.693/\beta$ where β is the slope of the terminal phase of the log of the concentration-time profile.

6.3.1.1.2 Plasma concentrations

For the intratracheal administration of TAP-lip and TAP-sol, average TA plasma concentrations (C_{PTA}) versus time profiles were fitted to a one compartment body model with first order absorption using the extended least-squares nonlinear regression method (SCIENTIST, Micromath, Salt Lake City, UT). For the intravenous administration of TAP-lip, plasma TA concentrations were fitted to a two compartment body model using the same program. Plasma concentrations after IT instillation of TAP-lip or TAP-sol and upon IV administration of TAP-lip were fitted to equations 6.4 and equation 6.1 respectively.

$$C_{PTA} = A\,(e^{-ke\,t} + e^{-ka\,t}) \qquad \text{(eq.6.4)}$$

where $A=D\,k_a/Vd\,(k_a-k_e)$ is the concentration of drug in the body at time zero, k_a is the first order absorption rate constant, k_e is the first order elimination rate constant.

Other pharmacokinetic parameters such as AUC and terminal half-life were determined as described above.

6.3.1.2 Non-compartmental pharmacokinetic analysis

The non-compartmental analysis for both plasma and lung concentrations over time was performed using standard techniques. The time to peak C_{PTA} (T_{max}) and peak C_{PTA} (C_{max}) was determined from the average plasma concentrations (C_{PTA}) versus-time profiles for both treatments, TAP-lip IT and TAP-sol IT.

Mean residence time (MRT) for average lung data (TAP-lip IT, TAP-lip IV and TAP-sol IT) and for TAP-lip IV plasma data was calculated from the relationship:

$$MRT_{0\to\infty} = AUMC_{0\to\infty} / AUC_{0\to\infty} \qquad \text{(eq. 6.5)}$$

where $AUMC_{0\to\infty}$ is the area under the first moment curve, and $AUC_{0\to\infty}$ is the area under the curve calculated as stated in equation 6.3. The area under the first moment $C_{TA} * t$ versus time curve ($AUMC_{0\to t}$) was calculated by the trapezoidal rule. Interpolation to infinity ($AUMC_{t\to\infty}$) was done by adding the ratio of the last $C_{TAL}*t$ measured and the first-order rate constant of the terminal phase and the ratio of the last measured concentration and the squared of the rate constant of elimination ($C_{TAL}*t/k_e + C_{TAL}/k_e^2$) .

Mean residence times for TAP-sol and TAP-lip IT plasma data were calculated from the relationship:

$$MRT = MRT_{IV} + MAT \qquad \text{(eq. 6.6)}$$

assuming that $MRT_{IV} = MRT_{\text{TAP-sol IT}}$ lung data. This assumption was based on the fact that glucocorticoids in solution are rapidly absorbed into the systemic circulation upon intratracheal instillation [117]. Mean absorption time (MAT) was calculated as $1/k_a$, where k_a is the absorption rate constant, which in the case of TAP-lip IT (flip-flop case), was equal to the calculated rate constant of the terminal phase (k_e).

6.3.2 Pharmacodynamic Analysis

Glucocorticoid receptor occupancy was considered as a pharmacodynamic end point. The cumulative receptor occupancy over time in lung ($AUC_{E\ lung}$) and liver (AUC_{Eliver}) upon IV administration of TAP-lip, IT instillation of TAP-lip and IT administration of TAP-sol was reported previously (chapter 5). TA lung and plasma concentrations were correlated to the pharmacodynamic effects (the percentage of lung

occupied receptors and the percentage of liver occupied receptors respectively) via a E_{max} model:

$$E = E_{max} * C/(EC_{50} + C) \qquad \text{(eq. 6.7)}$$

E_{max} is the maximal response (maximum receptor binding), EC_{50} is the pseudo-steady-state drug concentration causing 50% of E_{max} and C is either TA lung concentration (C_{TAL}) or TA plasma concentration (C_{PTA}).

A confidence interval (95%) based test was used to test for significant differences in EC_{50} values among the treatments.

6.4 Results

6.4.1 Pharmacokinetics of TA After Intratracheal Administration of TAP-lip

The average TA plasma and lung concentrations after IT administration of TAP-lip are shown in Figure 6.1 and 6.2 respectively. The pharmacokinetic parameters resulting from the analysis of plasma and lung data are listed in Tables 6.1 and 6.2 respectively. Non-linear curve fitting of the average plasma levels indicated that the fall in plasma concentrations following IT administration of TAP-lip, were best described by a monoexponential decline with first order absorption, while lung concentrations were depicted by a biexponential decay. The evaluation of goodness of fit was done by comparing the respective model selection criteria (MSC). This modified Akaike information criterion allows the comparison of the appropriateness of a model [118]. MSC values for lung and plasma concentrations were 1.1 and 0.1 respectively.

Significant differences in the rate constant of the terminal phase obtained for TAP-lip IT and TAP-sol IT plasma data (see assumption below), suggested the presence of a

"flip-flop" case for the average plasma levels following TAP-lip IT. The $AUC_{0\to\infty}$ for the drug plasma concentration-time profile was 226 ng h/ml, the terminal half-life was 4.1 hr. Maximum TA levels of 28 ng/ml were reached at 5 h after administration. The mean absorption time (MAT) and MRT were 5.8 h and 7.5 h respectively.

For the average lung concentration-time profile, the $AUC_{0\to\infty}$ was 6.3 μg h/g lung, the half-life for the terminal phase was 5.8 h. The mean residence time was 6.4 hr.

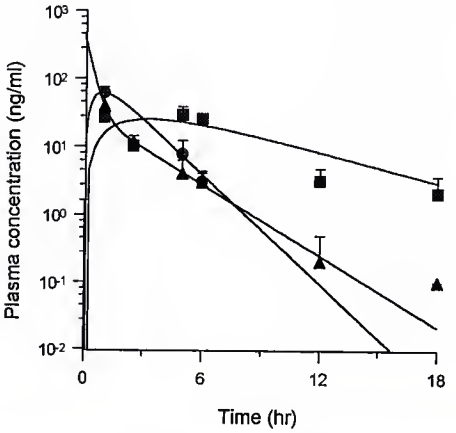

Figure 6.1. Average plasma concentrations (mean ± SD) of triamcinolone acetonide after intratracheal administration of TAP-lip (■), intravenous administration of TAP-lip (▲), and intratracheal administration of TAP-sol (●). (TAP-lip 100 μg/kg). n= 3 to 6.

6.4.2 Pharmacokinetics of TA After Intravenous Administration of TAP-lip

The average plasma and lung concentrations after IV administration of TAP-lip are shown in Figures 6.1 and 6.2, respectively. The pharmacokinetic parameters resulting from the analysis of plasma and lung data are listed in Tables 6.1 and 6.2, respectively.

Average plasma and lung concentration-time profiles were best described by a two compartment open model (MSC 5.6 and 4.3, respectively). The $AUC_{0\to\infty}$ for the drug plasma concentration-time profile was 102 ng h/ml, the half-life for the terminal phase was 1.7 h. The mean residence time was 2.3 h.

For the lung concentration-time profile, the $AUC_{0\to\infty}$ was 0.18 µg h/g lung, and the half-life for the terminal phase was 2 hr. The mean residence time was 2.9 hr.

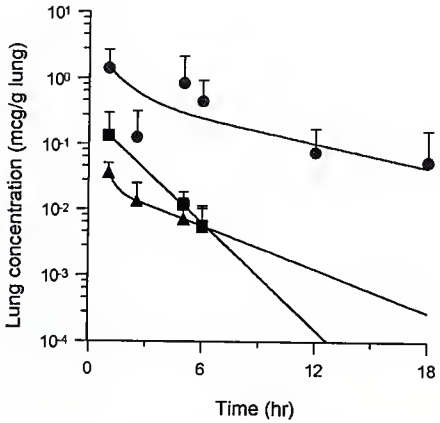

Figure 6.2. Average lung concentrations (mean ± SD) of triamcinolone acetonide after intratracheal administration of TAP-lip (●), intravenous administration of TAP-lip (▲), and intratracheal administration of TAP-sol (■). (TAP-lip 100 µg/kg). n= 3 to 6.

6.4.3 Pharmacokinetics of TA After Intratracheal Administration of TAP-sol

The average plasma and lung concentrations after IT administration of TAP-sol are shown in Fig. 6.1 and 6.2 respectively. The pharmacokinetic parameters resulting from the analysis of plasma and lung data are listed in Tables 6.1 and 6.2 respectively. The average

plasma concentration-time profile was best described by a one compartment open model with first order absorption (MSC=7.5), while average lung concentrations were depicted by a one compartment open model (MSC=8.8). The $AUC_{0\rightarrow\infty}$ for the drug plasma concentration-time profile was 179 ng h/ml, the terminal half-life was 1.1 h. Maximum TA levels of 62 ng/ml were observed after 1 h. The mean residence time was 1.6 h.

For the average lung concentration-time profile the $AUC_{0\rightarrow\infty}$ was 0.4 μg h/g lung. The terminal half-life obtained for lung data was 1.1 h, identical to that obtained when analyzing plasma data. [117] have reported that the absorption of glucocorticoids in solution after IT administration occurs unhindered. Therefore, we assumed here that the intratracheal administration of TAP-sol is equivalent to its intravenous administration, and therefore, the resulting MRT value after intravenous administration of TAP-sol is equal to MRT after intratracheal administration of the drug in solution (TAP-sol IT), which is 1.6 hr.

The AUC_{lung}:AUC_{plasma} after TAP-lip IT (Table 6.2) was 28 higher than that obtained after the intratracheal administration of a control solution, indicating the superiority of the liposomal formulation to act as a depot to supply the drug continuously to the site of action.

Table 6.1 Pharmacokinetic parameters for average triamcinolone acetonide plasma concentrations after intratracheal instillation (IT) of TAP-lip, intravenous administration of TAP-lip and intratracheal administration of TAP-sol.

	TAP-lip IT administration	TAP-lip IV administration	TAP-sol IT administration
Compartmental analysis			
A (ng/ml)	69.0	390.0	166.0
B (ng/ml)		32.0	
β (hr^{-1})		0.41	
$AUC_{0\rightarrow\infty}$ (ng.h/ml)	226	102	179
$t_{½}$ β (h)		1.7	
$t_{½}$ (hr)	4.1		1.1
k_a (hr^{-1})	0.5		2
k_e (hr^{-1})	0.17		0.6
Non-compartmental analysis			
C_{max} (ng/ml)	28		62
t_{max} (hr)	5		1
MAT (hr)	5.8		
MRT (hr)	7.5	2.3	1.8

Table 6.2. Pharmacokinetic parameters for average triamcinolone acetonide lung concentrations after intratracheal instillation (IT) of TAP-lip, intravenous administration of TAP-lip and intratracheal administration of TAP-sol.

	TAP-lip IT administration	TAP-lip IV administration	TAP-sol IT administration
Compartmental analysis			
A (µg/g lung)	2.3	0.42	0.25
B (µg/g lung)	0.54	0.025	
α (hr^{-1})	0.9	3.2	
β (hr^{-1})	0.12	0.35	
$AUC_{0\rightarrow\infty}$ (µg h/g lung)	6.3	0.15	0.37
$t_{½}$ β (hr)	5.8	2.0	
$t_{½}$ (hr)			1.1
k_e (hr^{-1})			0.6
AUC_{Lung} :AUC_{plasma}	28	1.5	2.1
Non-compartmental analysis			
MRT (hr)	6.4	2.9	1.6

6.4.4 Pharmacodynamics

In order to test for potential assay artifacts, such as *ex vivo* release of TAP from intact liposomes followed by its cleavage to TA during the thawing of homogenates for drug quantification, experiments were performed in which lung homogenates from untreated animal were spiked with either TAP-lip (TAP 100 μg) or TA in solution (TA 100 μg). The resulted mixtures were frozen overnight and consequently the amount of TA analyzed by HPLC as described in the methods section. We found a 30 % ± 12 (n=3) release of TAP from liposomes after freezing and thawing of the samples, indicating that the amount of sodium molybdate (potent phosphatase inhibitor) was not sufficient to prevent the cleavage of TA.

Table 6.3 Pharmacodynamic data obtained from curve fits of TAP-lip IT, TAP-lip IV, and TAP-sol IT average data. *Data reproduced from chapter 5.

	TAP-lip IT administration	TAP-lip IV administration	TAP-sol IT administration
E_{max} (%) Plasma Data	92	87	80
EC_{50} (ng/ml) Plasma Data	17	6	3
E_{max} (%) Lung Data	89	93	90
EC_{50} (ng/g lung) Lung Data	200	9	10
*Mean pulmonary effect time MET (hr)	5.7	3.8	3.0

The effect (receptor occupancy) versus concentration profiles after TAP-lip IT, TAP-lip IV and TAP-sol IT, are illustrated in Figures 6.3 and 6.4 for the local (lung) and systemic effects (liver), respectively. The pharmacodynamic parameters for the three modalities are listed in Table 6.3. The EC_{50} plasma values for TAP-lip IT, TAP-sol IT and TAP-lip IV were 17 ± 7.0, 6 ± 1.2, and 3 ± 1.3 ng/ml, respectively, indicating no significant difference among them. For the lung data, 2 subgroups of EC_{50} were observed, a high value of 200 ± 77 ng/g lung, obtained after TAP-lip IT and a small value of 9 ± 3 ng/g lung and 10 ± 4 ng/g lung, obtained after TAP-lip IV and TAP-sol IT, respectively. The significant difference among this group (lung data) is likely due to the release of TAP from intact liposomes and subsequent cleavage to TA, during thawing of samples.

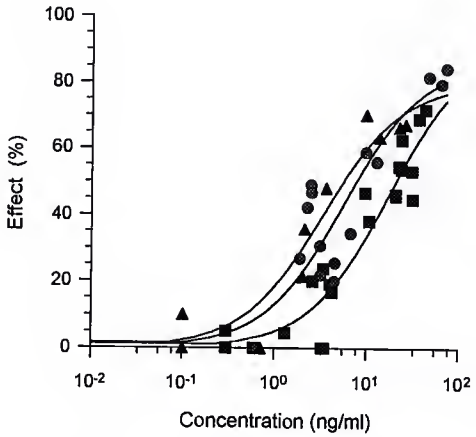

Figure 6.3. Systemic effects (% liver receptor occupancy; mean ± SD) as a function of triamcinolone acetonide (plasma concentrations) after intratracheal administration of TAP-lip (■), intravenous administration of TAP-lip (●), and intratracheal administration of TAP-sol (▲). (TAP-lip 100 μg/kg). n= 3 to 6.

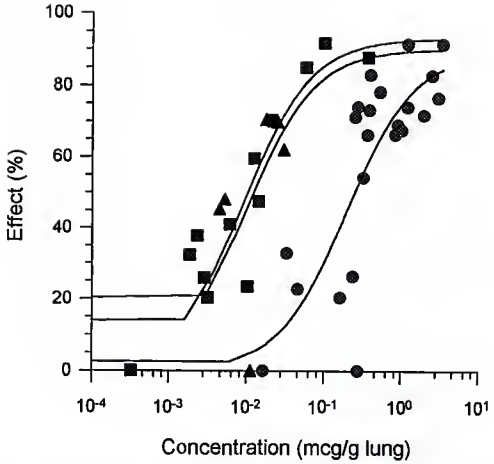

Figure 6.4. Local effects (% lung receptor occupancy; mean ± SD) of triamcinolone acetonide (lung concentrations) after intratracheal administration of TAP-lip (●), intravenous administration of TAP-lip (■), and intratracheal administration of TAP-sol (▲). (TAP-lip 100 μg/kg). n= 3 to 6.

6.5 Discussion

In the present study we have shown that the encapsulation of triamcinolone acetonide phosphate (a prodrug of triamcinolone acetonide) in liposomes favorably alters its pharmacokinetics, with no indications for changes in the pharmacodynamic interactions between glucocorticoid and receptor.

Based on our studies, the average lung concentration-time profile after intratracheal instillation of TAP-lip showed a biexponential decay (Figure 6.2). A similar biphasic decline was observed after pulmonary instillation of liposomal Ara-C [119]. As shown in chapter 7, we found in *in vitro* experiments that the efflux of 6-carboxyfluorescein from liposomes (with the same lipid composition and size as TAP-lip)

in the presence of surfactant was also biphasic. Therefore, it is likely that the biphasic decline observed after instillation of liposomes is due to a differential interaction of liposomes with the surfactant pool present in the airways. In contrast, the biexponential decay observed for the plasma concentration after the intravenous administration of TAP-lip has been well characterized. Gregoriadis and Neerunjun [80] observed that the biphasic decay pattern of intravenously injected liposomes may be due to the presence of two elimination pathways: a faster one, due to a saturable uptake by the reticuloendothelial system (RES), and a slower one, due to uptake by the liver parenchymal cells.

Burton and Shanker [117] have reported that the absorption of glucocorticoids in solution from the airways occurs rather fast (dexamethasone half-life of absorption = 1.7 minutes). Based on these observations, we assumed that the pharmacokinetics of TAP-sol administered IT is equivalent to an intravenous dose of TAP-sol. The plasma half-life following TAP-lip IT was significantly longer (4.1 hr; Table 6.1) than the one obtained after TAP-sol IT (1.1 hr). This occurs when absorption rather than elimination dictates the decline in plasma concentrations, indicating the presence of a "flip-flop" case and therefore, slow absorption into the systemic circulation. The intratracheal administration of TAP-lip also resulted in a longer pulmonary half-life (6 hr) than that after TAP-sol (1.1hr). These two observations show that liposome-encapsulated glucocorticoids are retained in the lung for a long period. A number of less lipophilic glucocorticoids (such as methyl prednisolone, and flunisolide) are rapidly absorbed from the airways because of their fast dissolution rate. This suggests that liposomes may be used as a means of enhancing retention of fast dissolving glucocorticoids in the respiratory airways.

We have shown in chapter 5, that the intratracheal administration of TAP-lip resulted in a longer mean pulmonary effect time than that after TAP-sol IT or after the intravenous administration of the liposomal formulation (see also MET Table 6.3 in this chapter). In the present studies, the intratracheal administration of triamcinolone acetonide phosphate encapsulated in liposomes (TAP-lip IT) resulted also in longer mean residence times for both lung and plasma data (Table 6.1 and 6.2), than the IT administration of TAP-sol or IV administration of TAP-lip. Thus, a change in the pharmacokinetics of the drug resulted in a change in its pharmacodynamics (overall effects). These longer MRT which are linked to a slow absorption into the systemic circulation also suggest that the design of once a day dosing may be possible leading to higher patience compliance.

Several studies have reported on the pulmonary half-life after intratracheal instillation of liposomes [113, 119]. In these studies, the pulmonary half-lives of the drugs in liposomes (with different chemical composition and sizes) varied from 4 hr to 18 hr compared to control solutions with half-lives less than 1.4 hr. These findings agree with our results as they demonstrate the potential of liposomes to dramatically modulate the pharmacokinetics. For example, Fielding and Abra [113] found that by including cholesterol in the liposomal formulation, the half-life increased from 1.4 hr to 18 hr. It is likely that the wide range in half-lives obtained after liposomal delivery is due to the differential size and chemical composition of the vesicles. These studies illustrate the possibility to further enhance the retention of TA in the airways by including cholesterol in our liposomal formulation.

AUC for plasma data after TAP-lip IT was 226 ng h/ml compared to 102 ng h/ml after TAP-sol IT. Assuming that the same dose of TAP was administered, theoretically, no

AUC for plasma data after TAP-lip IT was 226 ng h/ml compared to 102 ng h/ml after TAP-sol IT. Assuming that the same dose of TAP was administered, theoretically, no differences should have been found. The reason for such a discrepancy may be the infrequency of sampling at early time points which underestimates the concentrations of TA after TAP-sol administration.

Compared to the drug in solution (TAP-sol IT), liposome-encapsulated TAP after IT showed a 28 times higher AUC_{lung}:AUC_{plasma} drug ratio (Table 6.2). However, as it will be mentioned below, the AUC_{lung} after TAP-lip IT represents a mixture of TA released from liposomes *in vivo* and TA released from liposomes during thawing of samples prior to the drug analysis. Despite this fact, these results show the superiority of the liposomal formulation in providing a drug depot at the site of action. The intravenous administration of TAP-lip resulted in a lung: plasma ratio of 1.5 (Table 6.2), indicating only a slight accumulation of drug in the lung cytosol after this route of administration. In addition, the intratracheal administration of TAP-sol resulted in a similar AUC_{lung}:AUC_{plasma} ratio of 2.1, suggesting that the higher AUC_{lung}:AUC_{plasma} ratio obtained after TAP-lip is linked to the pulmonary administration of liposomal TAP. An analysis of single lung:plasma drug ratio values after TAP-sol (data not shown) administration were constant over time and close to two, indicating that the extent of protein binding in both lung cytosol and plasma tissue was nearly similar.

Considering the complexity of the assay, no significant difference in EC_{50} values (Table 6.3) were obtained for lung and plasma data after TAP-sol IT and TAP-lip IV. The EC_{50} values for lung data after TAP-lip IT (200 $\pm$ 77 ng/g lung) were significantly greater (20 times higher than that after TAP-sol IT (10 $\pm$ 4 ng/g lung) . This significantly higher

EC_{50} value is unlikely to be associated with changes in the pharmacodynamics. Instead, there is a high possibility that such differences may be due to assay artifacts, since control studies showed a 30 % ± 12 conversion of TAP (release from liposomes during the freezing and thawing process) to TA, and consequently the measured concentrations included the inactive TAP which resulted in a shift of the concentration-response curve to the right (Figure 6.4). It is clear that the concentration of sodium molybdate, a potent protease inhibitor [120] used in the present study was not sufficient to stop the cleavage. This consistency in the intrinsic pharmacodynamic properties after liposomal administration was expected, since there is no link between pharmacokinetics and EC_{50} (receptor affinity).

It has been stated [100] that the overall effects of a drug (effect-time profile) depend not only on the pharmacokinetics of the drug (concentration-time relationship), but also on its intrinsic pharmacodynamic properties (e.g. receptor binding affinity), in such a way that changes in the overall effects may be due to changes in the pharmacokinetics or in the pharmacodynamics. The results of the present study indicate that the pulmonary administration of glucocorticoid-encapsulated liposomes favorably alters its pharmacokinetics with no strong indication for changes in its intrinsic pharmacodynamic properties (such as EC_{50}, E_{max}). Thus, the observed changes in the overall effects (prolonged receptor occupancy) were because of changes in the pharmacokinetic properties of the drug. The pulmonary administration of TAP-lip resulted in a higher mean residence time, higher lung:systemic drug ratio as well as in more prolonged pulmonary effects, which are superior to a control solution of the drug administered in the same fashion and which may be superior to the currently available

glucocorticoid aerosol formulations. The observed change in pharmacokinetics for TA is likely to be responsible for the increase in targeting. While future work is needed to further improve the assay as well as sampling times, the results of the present study suggest the suitability of liposomes to increase the retention of glucocorticoids in the airways.

CHAPTER 7
EFFECT OF DOSE AND RELEASE RATE ON PULMONARY TARGETING OF GLUCOCORTICOIDS USING LIPOSOMES AS A MODEL DOSAGE FORM

7.1 Introduction

Inhaled glucocorticoids are the drugs of choice for the treatment of chronic asthma, because they attenuate locally the underlying inflammatory process [16]. Aerosolized glucocorticoid delivery to the lungs has potentially great clinical and therapeutic advantages. However, it is increasingly recognized that systemic side effects off-set their putative advantages due to the necessary frequent administration of inhaled drugs [39, 121]. Hence, technologies to enhance pulmonary targeting of inhaled glucocorticoids are desired.

Different approaches have been taken in order to optimize pulmonary targeting. These include drugs with high systemic clearance and low oral bioavailability [51]. Alternatively, sustained release formulations, such as liposomes, have also been suggested to prolong pulmonary drug action and reduce systemic side effects (anticancer drugs [122]; sodium cromoglycate [123]; glucocorticoids [23, 99]). Other approaches include theoretical models which simulate the fate of drugs in the airways upon inhalation. For example, Byron 1986 [39], and Gonda 1988 [54], developed pharmacokinetic models that predict the drug residence time in different regions of the respiratory tract upon aerosol

administration. These theoretical models have underscored the importance of certain physical and physicochemical factors (mode of inhalation, release kinetics, and particle size) as well as pharmacokinetic properties (pulmonary clearance, and absorption) of drugs as related to their suitability for pulmonary administration. Although very useful to improve dosage regimens, these simulations were not intended to quantify systemic effects, or to distinguish between local and systemic side effects.

Recently, we have used a mathematical model which predicts pulmonary selectivity of inhaled glucocorticoids by taking into account the pharmacokinetic/pharmacodynamic properties of these drugs. Extrapolations from this theoretical model suggest that drug release rate and dose are independent factors which can modulate the extent of pulmonary targeting.

In the present study, an animal model [56] was used to assess the effect of release rate and dose on pulmonary targeting of glucocorticoids upon intratracheal instillation of liposome preparations with different release rate characteristics and different doses of encapsulated material. Thus, this study provides experimental *in vivo* information on additional biopharmaceutical factors that should be considered in the development of aerosol dosage forms designed for optimal pulmonary targeting.

7.2 Material and Methods

7.2.1 Chemicals

Triamcinolone acetonide 21-phosphate dipotassium salt was donated by Bristol Myers Squibb, (Germany). {6,7-^{3}H(N)} triamcinolone acetonide (35.4 Ci/mmol) was purchased from DuPont NEN® Research Products (Boston, MA). Phospholipids (purity

>99%), 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and 1,2-distearoyl-sn-glycero-3-{phospho-rac-(1-glycerol)} (DSPG) were purchased from Avanti Polar Lipids (Alabaster, AL). All other reagents were of analytical grade.

7.2.2 Animals

Male Fisher-344 non-adrenalectomized rats weighing approximately 250 g were obtained and maintained as described in chapter 5.

7.2.3 Preparation of TAP-liposomes

A previously described method (see chapter 5) was slightly modified to prepare multilamellar vesicles containing triamcinolone acetonide phosphate (TAP). Chloroform solutions of DSPC (89.4 mg) and DSPG (10.1 mg) were placed in a round bottom flask. The organic solvent was removed under vacuum at 63 °C until a dry film was obtained and 1 ml of 60 mg/ml of TAP in isotonic phosphate buffer saline (Cellgro™) was added. Preliminary studies have shown that 60 mg/ml of TAP produces maximum encapsulation efficiency. The drug and lipids were dispersed mechanically for 1 hr at 63 °C, above the transition temperature (Tc) of the lipids. After 5 freeze/thaw cycles, the vesicles were extruded through 800 nm polycarbonate membrane filters (Poretics, Co., Livermore, CA) using a stainless-steel metallic extruder (Lipex Biomembranes Inc., Vancouver, B.C.). The resulting vesicles were separated from non-encapsulated drug by size exclusion chromatography (Sephadex G-50) and characterized for size (volume weighted mean diameter), encapsulation efficiency and osmolarity as described in chapter 5. The

encapsulation efficiency (%E) was expressed as percent moles of solute entrapped per mole of lipid (eq 7.1). The lipid content was determined as described below.

$$\%E = \frac{\text{Amount of TAP (mol)}}{\text{Amount of total lipid (mol)}} * 100 \qquad \text{(eq. 7.1)}$$

7.2.4 Preparation of 6-Carboxyfluorescein Liposomes

Liposomes containing entrapped 6-carboxyfluorescein (CF-liposomes) were prepared as described above for TAP-liposomes. CF was selected as a surrogate solute marker to be encapsulated in multilamellar vesicles because, like TAP, it is a water soluble substance and would be expected to partition similarly within the liposomal matrix. One ml of 200 mM 6-carboxyfluorescein (purified according to Ralston, 1981 [124], was added to the dried film of phospholipids. The mixture was dispersed, extruded through 200 nm or 800 nm polycarbonate filters and the resulting liposomes were processed and characterized as described for TAP-liposomes in chapter 5.

7.2.5 Lipid Determination

The lipid content of the liposomal preparations was determined by a colorimetric method [109] with minor modifications. Duplicates of 0.010 ml of the liposome dispersion were evaporated at 40 °C for 1 hr using a concentrator evaporator model RC10-10 (Jouan Inc., Winchester VA) followed by the addition of 3 ml of 0.1M ferric chloride hexahydrate ($FeCl_3.6H_2O$) and 0.4 M ammonium thiocyanate (NH_4SCN) in deionized water. The tubes were sonicated for 2 min, vortexed 4 times (15 seconds each time) and incubated at room temperature for 1 hr. Subsequently, 4 ml of chloroform were added; the tubes were closed

and vortexed 3 times (20 seconds each time). The samples were kept at room temperature until clear separation of the layers had occurred. The aqueous (upper) layer was removed and the optical density (absorbance) of the chloroform layer was measured at 488 nm. For the calibration curve, 0.01-0.1 ml aliquots of a mixture of 90% DSPC and 10% DSPG in chloroform (1 mg/ml total lipid) were assayed in duplicate.

7.2.6 *In vitro* Release Rate Studies

The effect of liposome size on the release rate of entrapped material was tested under sink conditions using 6-carboxyfluorescein as a marker and Triton X-100 (0.03%) as a leakage inducer.

Purified CF-liposomes (equivalent to 6 μM total phospholipid) were diluted 3-fold with PBS, followed by addition of Triton X-100 (0.03% final concentration). After vortexing, 200 μl aliquots of each sample were transferred into 96-well plates well and incubated at 37 °C. Carboxyfluorescein efflux was monitored over 5 hr on a luminescence spectrophotometer Perkin Elmer model LS50B with an excitation wavelength of 470 nm and emission wavelength of 520 nm. A blank value was obtained by carrying out the experiment in the absence of Triton X-100. The 100% fluorescence was determined after addition of 50 μl of Triton X-100 (0.03%) to each sample. The percentage of carboxyfluorescein released was calculated according to equation 7.2 [125],

$$I(\%) = 100 * \frac{(Ia - Ib)}{(Ix - Ib)} \qquad \text{(eq. 7.2)}$$

where Ix is the 100% fluorescence intensity value; Ia and Ib are the fluorescence intensities after incubation with and without surfactant, respectively.

The percentage of carboxyfluorescein encapsulated versus time was fitted to a biexponential equation (Scientist, Micromath, Salt Lake City, UT) and the half-life of release was calculated from the initial and terminal phase.

7.2.7 Animal Experiments

Animals were anesthetized via intraperitoneal injection and TAP 800 nm liposomes (TAP-lip 800 nm; equivalent to 100 μg/kg of TAP) were administered by intratracheal instillation as described in chapter 5. Animals (one animal per time point) were decapitated at 1, 2.5, 6, 12 or 18 hours after intratracheal (IT) administration of the preparation. The lungs and livers were immediately processed for receptor binding studies. A total of 3 independent experiments were performed on different days under the same conditions.

To test for the effect of dose on pulmonary targeting, similar experiments were performed using doses of 2.5, 7.5, 25, 100 or 450 μg/kg of TAP-lip 800 nm, respectively. The various doses were administered IT (3 series of experiments for each dose).

7.2.8 Receptor Binding Studies

A previously described receptor binding assay was employed (see chapter 4) with minor modifications. In the current study, one high concentration of ^{3}H-triamcinolone acetonide (30 nM ^{3}H-TA in the presence and absence of high concentrations of unlabeled TA, 3 μM) was used to determine the maximum specific binding (B_{max}). This was justifiable as the comparison of calculated B_{max} values using this method and the previously employed Scatchard method resulted in similar values for B_{max} (R^2=0.985, n=45) (section 4.2 chapter 4).

7.2.9 Data Analysis

A paired t-test was used to test for significant differences in receptor occupancy between lung and liver. The data set represented the pool of paired (hepatic and pulmonary) receptor occupancies of all individual single time points (not AUC's) included in a given experimental sub-set. Statistical significance was assumed for $p<0.05$.

To quantify the degree of pulmonary targeting, the cumulative change from baseline (AUC) was calculated for the 18 hour investigation period by the trapezoidal rule from percent occupied receptor (E_x)-time profiles for both the local and systemic organs. Pulmonary targeting (PT) for TAP-lip 800 nm as well as for previously reported data on TAP-lip 200 nm and TAP-sol (chapter 5) was defined as

$$PT=AUC_{Lung}-AUC_{Liver} \qquad \text{(eq. 7.3)}$$

For comparison, the previously employed targeting index ($PT=AUC_{Lung}/AUC_{Liver}$) was also derived.

Differences among TAP-lip 800 nm, TAP-lip 200 nm and TAP-sol and among doses, were tested for significance using Tukey's multiple comparison test assuming alpha<0.05.

The mean effect time (MET) was generally calculated from AUC_∞ and $AUMC_\infty$ according to equation 7.4 assuming that receptor levels returned to baseline during the observation period. The area under the first moment curve ($AUMC_\infty$), was determined for the pulmonary receptor occupancy by the trapezoidal rule from E_X*t_X versus t_X-pairs.

$$MET= AUMC_\infty/AUC_\infty \qquad \text{(eq. 7.4)}$$

In instances where the effect did not return to baseline, MET was calculated from AUC_t and $AUMC_t$, where t represents the time of last measurement.

7.3 Results

7.3.1 Characterization of liposomes

The volume weighted mean particle diameter (mean diameter) of the liposomal preparations resulting from the Gaussian distribution analysis was 220 ± 18 nm (nm=10) for the 200 nm CF-liposomes and 760 ± 90 nm (n=21) for the TAP-lip and CF-liposomes 800 nm. The lipid contents of TAP-lip and CF-liposome preparations were 0.092 mmol/ml ±0.013 (n=10) for the 200 nm liposomes and 0.10 mmol/ml ± 0.014 (n=21) for the 800 nm liposomes. This represents approximately 70% of the initial amount of lipid used (0.14mmol/ml). The encapsulation efficiency for TAP-lip 800 nm was 16.5 ± 2.1 % (n=18), which corresponds to molar drug/total lipid ratio of about 1:7.

7.3.2 *In vitro* Release Studies

A plot of log (% carboxyfluorescein encapsulated) versus time showed a biexponential decay for both liposome sizes (Figure 7.1). The CF-lip 200 nm released about 80% of CF with a half-life of 23 min while the remaining 20% was released with a half-life of 3.7 hr. CF-lip 800 nm showed a fast initial phase for approximately 10% of the CF ($t_{½}$ =4 min) with a more prolonged terminal phase ($t_{½}$ =9.0 hr) for the remaining 90% of CF released.

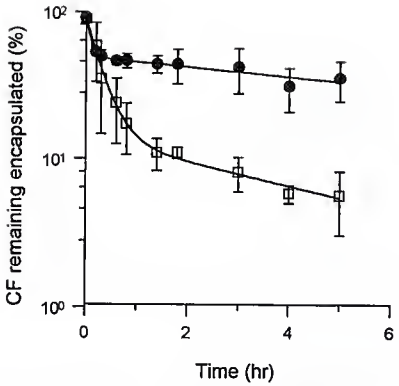

Figure 7.1. *In vitro* release studies of DSPC:DSPG CF-liposomes at 37 °C under sink conditions as a function of liposome size: 200 nm liposomes □, 800 nm liposomes ●. The percentage of 6-carboxyfluorescein remaining encapsulated over time of incubation is shown. Error bars represent mean ± S.D. for n=3

7.3.3 Receptor Binding

Time courses of free glucocorticoid receptors in the lung and liver after intratracheal administration of 100 μg/kg TAP-lip 800 nm are compared in Figure 7.2 to previous data obtained for equivalent doses of TAP in solution (TAP-sol) and TAP in 200 nm liposomes (TAP-lip 200 nm lip) (chapter 5).

Pulmonary targeting (PT), defined as the difference between cumulative lung and liver receptor occupancies, was most pronounced for the slowest release preparation ($PT_{TAP\text{-}lip\ 800\ nm}$=379 %*h, $p<0.02$), followed by the 200 nm preparation ($PT_{TAP\text{-}lip\ 200nm}$=150 %*h $p<0.02$). No pulmonary targeting was observed for TAP in solution ($PT_{TAP\text{-}sol}$=28 %*h $p>0.2$; Figures 7.2 and 7.3). Multiple comparison using the Tukey's test showed significant difference

among these preparations. The mean effect times (MET; refer to Table 7.1) correlated well with the *in vitro* release studies; these studies demonstrated that with increasing liposome size, both *in vitro* stability (see release kinetics Figure 7.1) and mean effect time were increased.

The administration of escalating doses of TAP-lip 800 nm revealed a distinct bell shape relationship between the TAP dose and PT with a maximum pulmonary targeting occurring at 100 μg/kg ($PT_{TAP\text{-}lip\ 800\ nm}$=379%*h) (Figures 7.4 and 7.5, Table 7.2). There were significant differences in receptor occupancies between organs for all doses except for 2.5 μg/kg and 450 μg/kg. Tukey's multiple comparison test applied to the means of pulmonary targeting as a function of dose revealed significant difference between 100 μg/kg and the rest of the doses. However, no significant difference was found among 7.5, 25 and 450 μg/kg, but between these three doses and 2.5 μg/kg.

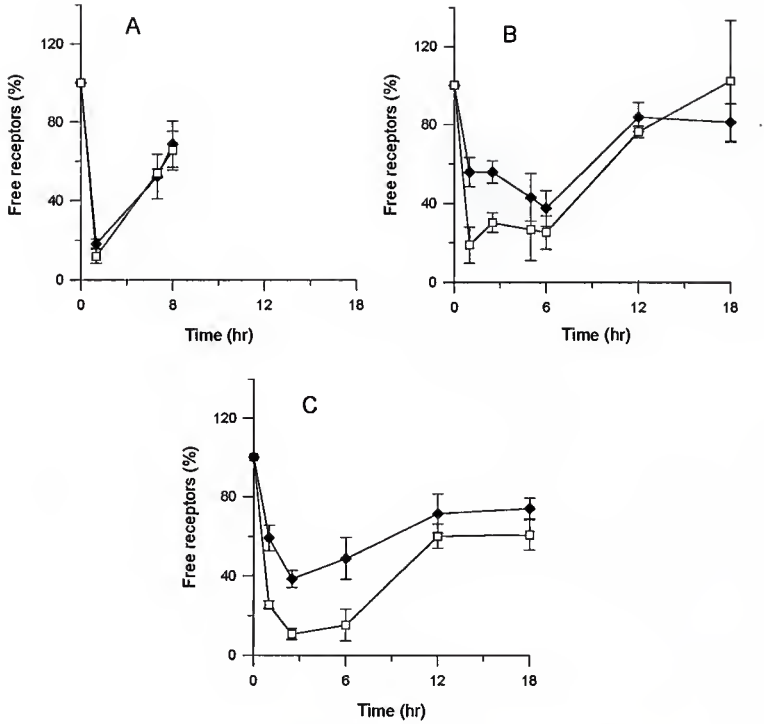

Figure 7.2. Lung □ and liver ◆ glucocorticoid receptor occupancy profiles after intratracheal instillation of 100 μg/kg of: A) TAP-sol (from chapter 5); B) TAP-lip 200 nm from (from chapter 5); and C) TAP-lip 800 nm. Error bars represent mean ± S.D. for n= 3 to 6

Table 7.1. Cumulative receptor occupancy (AUC), pulmonary targeting and mean pulmonary effect times (MET) after intratracheal administration of TAP in solution (TAP-sol, immediate release; 100 μg/kg), TAP in 200 nm liposomes (TAP-lip 200 nm, intermediate release; 100 μg/kg) and TAP in 800 nm liposomes (TAP-lip 800 nm, slow release; 100 μg/kg).

	TAP-sol*	TAP-lip 200 nm*	TAP-lip 800 nm
	Cumulative change from baseline (AUC %*h)		
LUNG	350	770	1070
LIVER	340	620	700
Pulmonary Targeting (%*h) (AUC_{lung}-AUC_{liver})	28	150	370
Pulmonary Targeting (AUC_{lung}/AUC_{liver})	1.0	1.2	1.5
Mean Pulmonary Effect Time (h)	3	5.7	>6.2

*Reproduced from chapter 5

Table 7.2. Cumulative receptor occupancy (AUC), pulmonary targeting and mean pulmonary effect times (MET) after intratracheal administration of escalating doses of TAP in 800 nm liposomes.

DOSE (μg/kg)	2.5	7.5	25	100	450
	Cumulative change from baseline (AUC %*h)				
LUNG	92	338	345	1074	1261
LIVER	78	111	130	696	1080
Pulmonary Targeting (%*h) AUC_{lung}-AUC_{liver}	14	227	213	379	176
Pulmonary Targeting (%*h) AUC_{lung}/AUC_{liver}	1.2	3.0	2.7	1.5	1.2
Mean Pulmonary Effect Time (h)	2.5	3.9	4	>6.2	>8.1

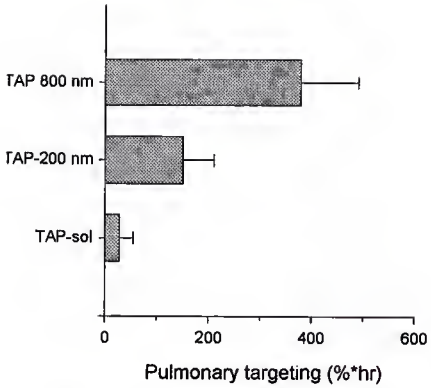

Figure 7.3. Pulmonary targeting as a function of drug release rate: TAP-sol (immediate release), TAP-lip 200 nm (intermediate release), TAP-lip 800 nm (slow release). Statistical significance was assumed for alpha < 0.05.

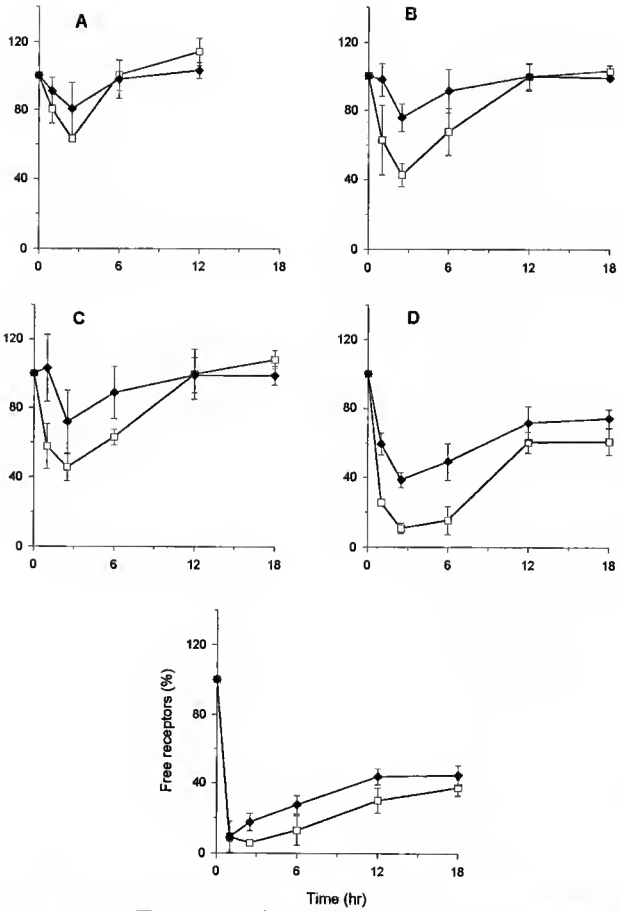

Figure 7.4. Lung □ and liver ◆ glucocorticoid receptor occupancy profiles after intratracheal instillation of escalating doses of TAP in 800 nm liposomes: A) 2.5 μg/kg, n=4; B) 7.5 μg/kg, n=3; C) 25 μg/kg, n=3; D)100 μg/kg, n=3; and E) 450 μg/kg, n=3. Error bars represent mean ± S.D.

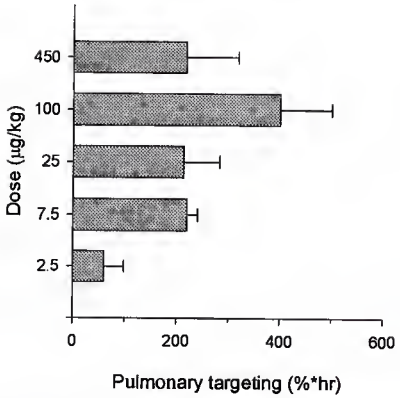

Figure 7.5. Pulmonary targeting as a function of escalating doses of TAP in 800 nm liposomes. Error bars represent mean ± S.D.

7.4 Discussion

The mathematical model [1] used here incorporates pharmacokinetic and pharmacodynamic properties of a drug after inhalation, and thereby allows prediction of pulmonary and systemic effects, and the effects of pharmacokinetics on pulmonary selectivity (in preparation). Based on theoretical simulations, the model suggested that, by altering the drug release rate or by changing the dose deposited in the lung pulmonary targeting can be significantly modulated. In the present study, we were interested in showing experimentally the relevance of these two factors to pulmonary targeting *in vivo*. For this purpose, pulmonary targeting was assessed using an animal model [56]. This model allows the determination of pulmonary targeting by simultaneously monitoring receptor occupancy in local (lung) and systemic organ (liver). We have reported in chapter 5 that pulmonary targeting can be calculated as the ratio of cumulative receptor occupancy

in lung versus liver (AUC_{Lung}/AUC_{Liver}). However, since computer simulations [56] have shown that pulmonary targeting defined in these terms reaches its maximum as dose approaches zero, we believe that pulmonary targeting is more accurately defined as the difference between lung and liver receptor occupancies (AUC_{Lung}-AUC_{Liver}), especially when pulmonary selectivity is evaluated as a function of dose (see Table 7.2 for comparison of both parameters).

In this study we selected liposomes as a model dosage form to show the relevance of dose and drug release rate on pulmonary targeting. Several studies using liposomes have shown that by altering the chemical composition and lipid content the *in vitro* and *in vivo* release kinetics of liposomes can be modulated [88, 126]. Based on our *in vitro* release studies, larger multilamellar liposomes (800 nm) released the encapsulated material with longer half-life than smaller vesicles (200 nm) (Figure 7.1). Size-dependent leakage from liposomes has been previously reported by Fielding [113], who pointed out that the drug release rate of liposomes following intratracheal administration is determined by the number of lamellae in the vesicles. Although it has been reported that upon aerosolization the stability of liposomes decreases as the initial vesicle size increases [104], this initial vesicle rupture (around 13 % for 800 nm liposomes) obtained for large vesicles may be beneficial since under these conditions a more rapid onset of drug action in the lung may be attained, followed by a prolonged release for the remainder of the dosage form.

Computer simulations as well as animal experiments have shown the advantage of controlling the drug release rate as a mean of increasing lung residence time upon intratracheal instillation [54, 113, 127]. In addition, some studies have suggested, but not demonstrated, that pulmonary selectivity depends on a slow release from the delivery form

[56, 23]. In this study, we have shown that the intratracheal (IT) administration of slow release triamcinolone acetonide phosphate liposomes (TAP-lip 800 nm) resulted in higher pulmonary selectivity (Figure 7.3) as well as in more prolonged pulmonary effects (Figure 7.2; see MET Table 7.1) than the IT administration of intermediate release rate liposomes (TAP-lip 200 nm) or an immediate release preparation (TAP-sol). Although pulmonary receptor occupancies increased relatively, liver receptor occupancy also increased as the release rate decreased (Table 7.1). This is not surprising since a slower absorption has been shown to increase the cumulative systemic effect for a given dose [100]. Our observations indicate that pulmonary selectivity was enhanced because the free (pharmacodynamically active) drug concentration in the lung exceeded that in the systemic circulation (liver) over a prolonged period of time when the drug release rate was slowed down. The clinical relevance of these findings is that by administering glucocorticoids in slow release preparations, the frequency of administration as well as the dose can be decreased. This will lead to reduction of systemic side effects while pulmonary effects are maintained. These findings also suggest that since currently available inhaled glucocorticoids differ in their dissolution rates [105-108], the pulmonary selectivity achieved by these drugs should also be different.

The administration of escalating doses of TAP-lip 800 nm showed a typical bell shaped curve with a maximum at 100 μg/kg (Figure 7.5), and a significant difference between pulmonary and hepatic receptor occupancies until a maximum was reached (Figure 7.4 panels B, C, D). Both the low (2.5 μg/kg) and high (450 μg/kg) doses of TAP resulted in close superimposition of lung and liver receptor occupancy and, consequently, in low pulmonary targeting (Figure 7.4A, 7.4E). Thus, these studies suggest

there is a dose "optimum" for which maximum pulmonary selectivity is observed (e.g. selectivity being defined in this case pharmacodynamically by the extent and duration of pulmonary glucocorticoid receptor occupancies). Although it is recognized that a dose optimum may not necessarily be directly indicative of clinical response in asthma of varying severity, our findings clearly show that overdosing and underdosing will always go parallel with a decreased pulmonary targeting. While speculative, this dose optimum concept may ultimately be of clinical importance: clearly, some asthmatics being treated with high doses of inhaled glucocorticoids might be prone to increasing systemic side effects at the expense of diminishing returns as regards pulmonary targeting.

Recent approaches to increase pulmonary selectivity of glucocorticoids have included the development of drugs with higher systemic clearance (such as budesonide [105]), and lower oral bioavailability (such as fluticasone propionate [128]), as well as the design of pulmonary delivery devices with increased pulmonary deposition (such as dry powder inhalers [129]). To our knowledge there have been no systematic attempts to increase pulmonary selectivity by optimizing drug release rate and dose. The animal experiments shown here together with previously reported computer simulations [1] illustrate the relevance of dose and release rate to enhance pulmonary targeting. These two factors should be considered in the development of drugs for the local treatment of airway diseases, as well as in the design of dosage regimens which would minimize systemic side effects

CHAPTER 8
ASSESSMENT OF PULMONARY SELECTIVITY OF TRIAMCINOLONE ACETONIDE AND FLUTICASONE PROPIONATE USING AN *EX VIVO* RECEPTOR BINDING ASSAY

8.1 Introduction

In the past decade, new inhaled glucocorticoids have been introduced for the treatment of asthma. These drugs tend to accumulate in the lungs, have a lower oral bioavailability, and a higher systemic clearance [23, 51, 53], factors that improve the benefit/risk ratio. Inhaled glucocorticoids also show greater receptor affinity and increased steroid potency [130], thus allowing clinical effects to be achieved at lower doses.

Triamcinolone acetonide is a potent glucocorticoid currently available in the United States for dermal and parenteral use and is marketed as a metered dose inhaler for use in asthma [131]. Fluticasone-17-propionate, has recently been approved in eight countries as a nasal spray for seasonal rhinitis and for the treatment of asthma. Fluticasone propionate which possesses the highest receptor affinity (eight times more than dexamethasone) and the lowest oral bioavailability (< 1 %) among inhaled glucocorticoids, is generally administered using a dry powder inhaler (diskhaler) [132].

We have shown in chapter 7 that pulmonary selectivity is affected by the pulmonary drug release rate. In addition, several studies have shown significant differences in the pulmonary absorption profile of commercially available inhaled glucocorticoids.

These studies give the basis to believe that the extent of pulmonary targeting achieved by these drugs is also different.

In vitro (induction of protease inhibitor [133]; receptor binding [56, 134]) and *in vivo* studies (skin-blanching [16]; inhibition of ear edema formation [135]) have reported the relative potencies of inhaled glucocorticoids at the site of action. However, these parameters do not provide information about the glucocorticoid pulmonary activity, as this is determined not only by the pharmacodynamics of the drug, but also by its pharmacokinetics. Some studies have shown differences on the extent of systemic exposure of inhaled glucocorticoids by determining plasma drug concentrations [136] and by monitoring the suppression of circulating lymphocytes and plasma cortisol [137], two markers for systemic side effects. However, these studies give no indication of pulmonary selectivity since it depends on both the topical as well as on the systemic effects.

Although the efficacy of inhaled glucocorticoids is well established, no studies have evaluated and compared their pulmonary selectivity because of quantification difficulties, mainly due to a lack of an appropriate surrogate marker for pulmonary effects.

The goal of the present study is to evaluate the pulmonary selectivity of two currently available inhaled glucocorticoids: triamcinolone acetonide and fluticasone propionate using the *ex vivo* animal model previously described and to compare them with data reported in chapter 5 for a glucocorticoid-encapsulated liposome formulation. In this study we have included not only the liver as a systemic organ to assess the extent of systemic absorption, but also the kidney and spleen, because of concerns on high intrinsic hepatic clearance on liver receptor occupancy.

8.2 Materials and Methods

8.2.1 Materials

Analytical grade chemicals were obtained from Sigma Chemical Co. (St. Louis, MO.); micronized fluticasone propionate was obtained as a gift from Glaxo Wellcome Research and Development Ltd. UK. Triamcinolone acetonide micronized was obtained from Bristol-Myers Squibb, Regensburg, Germany). Lactose monohydrate NF extra fine (particle size 10-30 microns) was obtained from EM industries Hawthorne, NY. {6,7-^{3}H(N)} triamcinolone acetonide (35.4 Ci/mmol) was purchased from DuPont NEN® Research Products (Boston, MA). All other reagents were of analytical grade.

8.2.2 Drug formulations

Fluticasone propionate was dissolved in a mixture of PEG 300 and ethanol (3:1 v/v) to obtain a final concentration of 200 μg/ml. This solution was used for the intravenous administration. Fluticasone propionate and triamcinolone acetonide were mixed with lactose to obtain a concentration of 4 μg/ mg lactose.

8.2.3 Animals

All animal procedures were approved by the Animal Care Committee of the University of Florida, an AAALAC approved facility. Specific-pathogen-free male F-344 rats, weighing approximately 250 g were housed in a 12 hr light/dark cycle, in a constant temperature environment. Animals were allowed free access to water and rat chow, but were food-fasted overnight prior to each experiment.

8.2.4 Drug Administration

8.2.4.1 Intratracheal instillation

The animals were housed in the operating room 12 h before the experiment to get them accustomed to the new environment. The day of the experiment the rats were handled gently to produce minimum stress. Once weighed, the rats were placed in a rat holder for intraperitoneal administration of the anesthetic (fresh preparation of the combination of 1.5 ml of ketamine 10%, 1.5 ml of xylazine 2% and 0.5 ml of acepromazine 1%) at the dose of 1 ml/kg. The depth of anesthesia was checked by tail pinch or pedal withdrawal reflex.

The neck of the completely anesthetized animal was shaved and aseptically cleaned with isopropyl alcohol 70%. A one centimeter medline vertical incision was made originating above the sternal notch. The neck muscles and glands were carefully dissected midline, until the trachea was exposed and a tracheotomy was performed between the third and fourth tracheal rings. A 1 3/8 inch tubing (butterfly 25 x 3/8 8" tubing, Abbott laboratories) attached to a delivery device for intratracheal administration of dry powders (Penn-Century, Philadelphia, PA), was introduced into the trachea. A mixture of 5 mg of extra fine monohydrate upgrade lactose and either fluticasone propionate (FP) or triamcinolone acetonide (TA) (4 μg/mg lactose) were placed in the chamber of the device and instilled in the lungs by insufflation of 3 ml of air into the delivery device. A sham rat was included in each set of experiments which received 5 mg of the vehicle (upgrade lactose). A total of four to seven independent experiments for FP and TA respectively, were performed for a given time point after IT administration

8.2.4.2 Intravenous administration

For the intravenous (IV) injection, animals were anesthetized as described above and the femoral vein exposed by dissection. Either 100µl of fluticasone propionate solution (200µg/ml) or 100µl of the vehicle (for the sham rat) were slowly injected using a tuberculin syringe with a 27 gauge needle. Animals (one animal per time point) including sham rat, were decapitated at 1, 3, 6, 12, or 18 hours after instillation. Lung, liver, spleen and kidneys were immediately processed for receptor binding studies. A total of four independent experiments were performed for a given time point after IV administration.

The skin was sutured using a cutting point 1/2 circle needle, and monofilament nylon 4-0 suture material. A simple interrupted suture pattern was used to close the incision. The animal was placed on its abdomen in a cage with rodent bedding under heat lamps and allowed to recover while observed (monitoring of the breathing rate by visual inspection of the chest wall).

8.2.5 Receptor Binding Studies

The receptor binding assay described in chapter 4 was employed with minor modifications. In the current study, one high concentration of ^{3}H-triamcinolone acetonide (30 nM ^{3}H-TA in the presence and absence of high concentrations of unlabeled TA, 3 µM) was used to determine the maximum specific binding (B_{max}). This was justifiable as the comparison of calculated B_{max} values using this method and the previously employed Scatchard method resulted in similar values for B_{max} (R^2=0.985, n=45) (chapter 4).

8.2.6 Data Analysis

A paired t-test was used to test for significant differences in receptor occupancy between lung:liver, lung:spleen, and lung:kidney. The data set represented the pool of paired receptor occupancies of all individual single time points (not AUC's) included in a given experimental sub-set. Statistical significance was assumed for $p<0.05$.

In order to quantify the degree of pulmonary targeting, the cumulative change from baseline (AUC_E) was calculated for the investigation period by the trapezoidal rule from percent occupied receptor (E_x)-time profiles for both the local and systemic organs. Pulmonary targeting was defined as

$$PT=AUC_{E\ Lung}-AUC_{E\ sys\ organ} \qquad \text{(eq 8.0)}$$

where $AUC_{E\ sys\ organ}$ represents the AUC_E of liver, spleen or kidney.

Differences in pulmonary targeting (defined as $AUC_{E\ Lung}$-$AUC_{E\ Kidney}$ or as $AUC_{E\ Lung}$-$AUC_{E\ Liver}$ or $AUC_{E\ Lung}$-$AUC_{E\ Spleen}$) within and among the treatments were tested for significance using Tukey's multiple comparison test assuming $\alpha<0.05$. Differences in pulmonary targeting (defined as $AUC_{E\ Lung}$-$AUC_{E\ Liver}$), between TA dry powder and triamcinolone acetonide phosphate encapsulated in 800 nm liposomes (TAP-lip 800 nm data reported in chapter 7), were tested using a t-test.

The mean pulmonary effect time was calculated from AUC_∞ and $AUMC_\infty$ according to equation 8.1. The area under the first moment curve (AUMC), was determined for the pulmonary receptor occupancy by the trapezoidal rule from E_X*t_X versus t_X-pairs. AUC_∞ and $AUMC_\infty$ were estimated by extrapolation to baseline assuming a linear decline of the effect over time at late time points [100].

$$MET = AUMC_{\infty}/AUC_{\infty} \qquad \text{(eq. 8.1)}$$

8.3 Results

8.3.1 Intratracheal Administration of Fluticasone Propionate (FP IT)

The cumulative receptor occupancies for the different organs are shown in Figure 8.1. There was a significant difference in receptor occupancy for lung versus liver, ($p<0.01$, Table 8.2) and for lung versus kidney ($p<0.04$), while no significant difference was found for lung versus spleen ($p>0.6$). Pulmonary targeting (PT), was most pronounced when lung and liver receptor occupancies were compared (PT=400 %*hr), followed by the pair lung/kidney (156 %*hr), while little or no targeting was observed for the pair lung/spleen (PT=115 %*hr; Table 8.1). Multiple comparison in pulmonary targeting among pairs were significant for lung/liver versus lung/spleen and lung/liver versus lung/kidney while no significant difference was obtained for lung/spleen versus lung/kidney (see Table 8.3). Mean effect time was 5.5 hr.

8.3.2 Intravenous Administration of Fluticasone Propionate (FP IV)

The cumulative receptor occupancies for the different organs are shown in Figure 8.2. There was a significant difference in receptor occupancy for lung versus liver ($p<0.1$) while no significant difference was found for the pairs lung versus kidney ($p>0.45$) and lung versus spleen ($p>0.4$). Pulmonary targeting was most pronounced for the pair lung versus liver (PT=289 %*hr), while little or no targeting was observed for the pairs

lung/spleen (PT=63 %*hr) and lung/kidney (PT=-40 %*hr) (Table 8.1). Multiple comparison in pulmonary targeting among pairs were significant for lung/liver versus lung/spleen and lung/liver versus lung/kidney while no significant difference was obtained for lung/spleen versus lung/kidney (see Table 8.3). Mean effect time was also 5.5 hr.

Table 8.1. Cumulative receptor occupancy (AUC_E), pulmonary targeting and mean pulmonary effect time after IT and IV administration of fluticasone propionate (FP, 100 μg/kg), IT administration of triamcinolone acetonide dry powder (TA, 100 μg/kg), IV administration of TA solution (100 μg/kg), and IT administration of triamcinolone acetonide phosphate in 800 nm liposomes (TAP-lip 800 nm) (100 μg/kg).

	FP pw IT adm	FP sol IV adm	TA pw IT adm	TA sol** IV adm	TAP-lip 800 nm IT adm*
	AUC E (% * h)				
LUNG	637	756	724	280	1070
LIVER	235	467	655	320	700
SPLEEN	522	693	716		
KIDNEY	481	797	609		
	Pulmonary targeting %*hr ($AUC_{E\,Lung}$ - $AUC_{E\,organ}$)				
($AUC_{E\,Lung}$ - $AUC_{E\,Liver}$)	400 ± 65	289 ± 60	70 ± 50	-40	370 ± 100
($AUC_{E\,Lung}$ - $AUC_{E\,Spleen}$)	115 ± 140	63 ± 120	10 ± 50		
($AUC_{E\,Lung}$ - $AUC_{E\,Kidney}$)	160 ± 60	-40 ± 180	115 ± 80		
Mean pulmonary effect time (h)	5.5	5.5	4.7		> 6.2

*Data reproduced from chapter 7. **Data reproduced from Hochhaus et al. 1995 [56].

Table 8.2 Statistical test (t test) for significance between tissues.

Treatment	($AUC_{E\ Lung}$-$AUC_{E\ Liver}$)	($AUC_{E\ Lung}$-$AUC_{E\ Spleen}$)	($AUC_{E\ Lung}$-$AUC_{E\ Kidney}$)
FP IT	0.01	0.6	0.04
FP IV	0.01	0.4	0.45
TA IT	0.02	0.9	0.02

Table 8.3 Multiple comparison (Tukey's test) for significance among pulmonary targeting values defined as ($AUC_{E\ Lung}$ - $AUC_{E\ Organ}$).

Treatment	lung/liver vs. lung/spleen	lung/liver vs. lung/kidney	lung/spleen vs. lung/kidney
FP IT	S ($p< 0.05$)	S ($p< 0.05$)	NS ($p> 0.05$)
FP IV	S ($p< 0.05$)	S ($p< 0.05$)	NS ($P>0.05$)
TA IT	NS ($p> 0.05$	NS ($p> 0.05$)	NS ($p> 0.05$)

8.3.3 Intratracheal Administration of Triamcinolone Acetonide (TA IT)

The cumulative receptor occupancies for the different organs are shown in Figure 8.3. There was a significant difference in receptor occupancy between lung versus liver ($p<0.02$) and lung versus kidney (0.02), while lung versus spleen receptor occupancy resulted in close superimposition ($p>0.9$). Pulmonary targeting was most pronounced for lung versus kidney (PT=115), followed by lung versus liver (PT=69) while no targeting was observed for lung versus spleen (PT=8) (Table 8.1). Multiple comparison in pulmonary targeting among pairs were no significant (see Table 8.3). Mean effect time was also 4.7 hr.

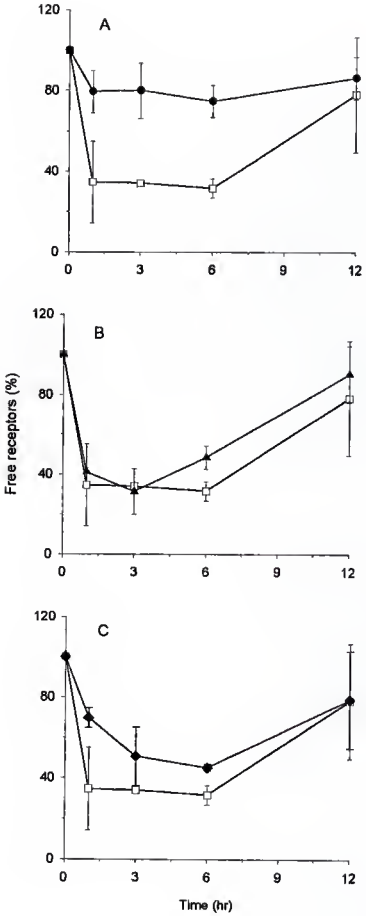

Figure 8.1 Receptor occupancy time profiles for : A) lung □ versus liver ●, n=3; B) lung □ versus spleen ▲, n=3; and C) lung □ versus kidney ◆ n=3, after intratracheal administration of fluticasone propionate-dry powder (100 μg/kg).

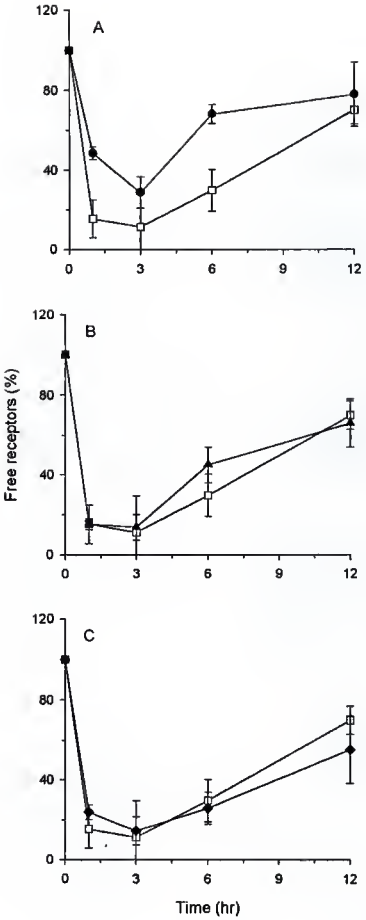

Figure 8.2 Receptor occupancy time profiles for : A) lung □ versus liver ●, n=3; B) lung □ versus spleen ▲, n=3; and C) lung □ versus kidney ◆ n=3, after intravenous administration of fluticasone propionate in solution (100 μg/kg).

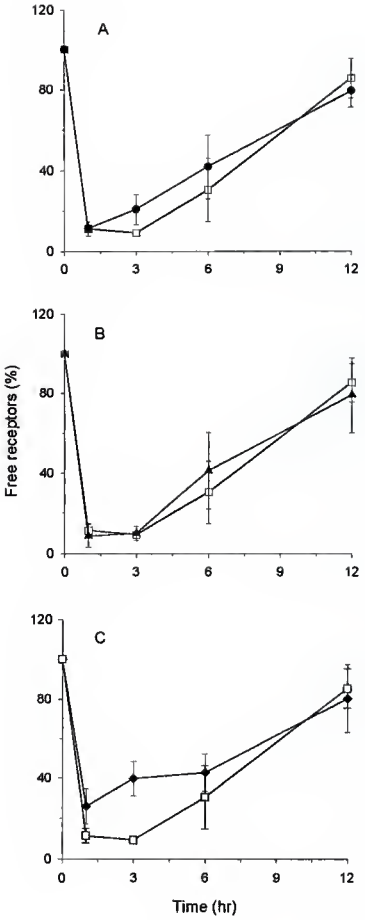

Figure 8.3 Receptor occupancy time profiles for: A) lung □ versus liver ●, n=6; B) lung □ versus spleen ▲, n=6; and C) lung □ versus kidney ◆ n=6, after intratracheal administration of triamcinolone acetonide-dry powder (100 μg/kg).

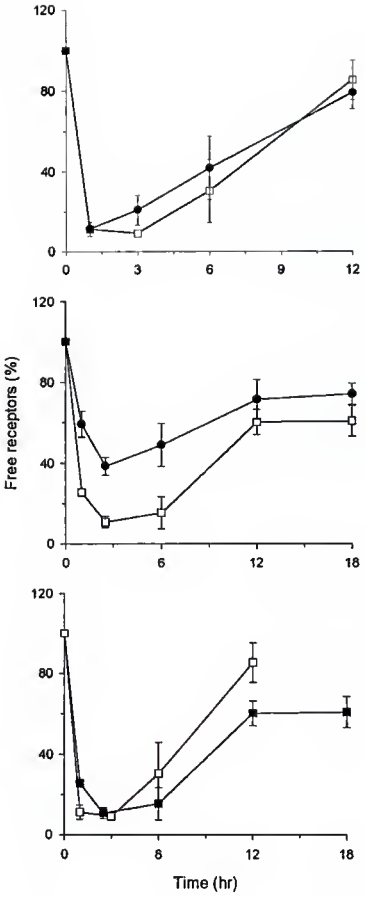

Figure 8.4 Lung □ and liver ● receptor occupancy time profiles for: A) Intratracheal administration of triamcinolone acetonide (TA)-dry powder (100 μg/kg); B) Intratracheal administration of TAP in 800 nm liposomes (TAP-lip 800 nm; TAP 100 μg/kg; reproduced from chapter 7) and C) Lung receptor occupancy for TA-dry powder □ and TAP-lip 800 nm ■

Multiple comparisons of pulmonary targeting (defined as $AUC_{E\ Lung}$-$AUC_{E\ Kidney}$) among the treatment groups using Tukey's test revealed significant differences between FP IV :FP IT and FP IV : TA IT, but not between FP IT : TA IT (see Table 8.3).

There was a significant difference in pulmonary targeting between equal doses of triamcinolone acetonide-dry powder and previously reported data (chapter 7) for triamcinolone acetonide phosphate encapsulated in 800 nm liposomes (TAP-lip 800 nm).

Mean pulmonary effect time (MET) was most pronounced for the liposome preparation (MET > 6.2 h) and least pronounced for triamcinolone acetonide administered as a dry powder (MET = 4.7; Table 8.1).

8.4 Discussion

The present study assessed the pulmonary selectivity of triamcinolone acetonide (TA), and fluticasone propionate (FP)-dry powders after intratracheal administration in male rats.

Several *in vitro* and *in vivo* studies have shown differences in the topical and systemic potency [135], receptor affinity [138, 139], and pharmacokinetic properties [22, 105, 107, 140] of inhaled glucocorticoids. In addition, pharmacokinetic/pharmacodynamic (PK/PD) models have been developed to assess the relationship between PK/PD and the extent of glucocorticoid systemic effects such as lymphocyte and cortisol suppression. [141]. These studies, however, gave no indication of the extent of pulmonary selectivity, as it is determined by both the extent of local, as well as systemic effects. Furthermore, pulmonary targeting has been rather difficult to evaluate because good surrogate markers for pulmonary effects are not available for human pharmacological studies. In this study

we used the *ex vivo* animal model previously described in chapter 3, to assess pulmonary selectivity of two commercially available inhaled glucocorticoids.

In chapters 5 and 7 we assessed pulmonary targeting by monitoring receptor occupancy in lung and liver. In the present study we have considered not only the liver as a marker for the extent of drug systemic exposure, but also tissues with no metabolic activity such as the kidney and spleen, because of concerns that the high intrinsic hepatic clearance of fluticasone propionate may affect the receptor binding results.

The intravenous (IV) administration of fluticasone propionate (FP) in solution resulted in similar cumulative receptor occupancies for lung ($AUC_{E\ lung}$=756 %*hr), kidney ($AUC_{E\ kidney}$=797 %*hr) and spleen ($AUC_{E\ spleen}$=693 %*hr). However, the liver receptor occupancy was significantly smaller ($AUC_{E\ liver}$=467 %*hr). This significant difference was not expected, since upon IV administration the free drug concentrations and thus, receptor occupancies should be the same in the entire body (assuming no tissue sequestration). The lower liver receptor occupancy observed is understandable, if one considers the high intrinsic hepatic clearance of FP. Because FP is highly metabolized in the liver, (which is also indicated by its oral bioavailability of lower than 1%), at any given time point, both free drug concentrations and liver receptor occupancies will be lower than those in the other organs. Contrary to this, recent studies [56] showed that the IV administration of TA, a glucocorticoid with lower intrinsic clearance, resulted in a close superimposition of lung and liver receptor occupancies (see Table 8.1). These findings suggest that the liver may not be an appropriate organ to assess the extent of systemic absorption of drugs with high intrinsic hepatic clearance such as fluticasone propionate. These results also suggest that, the assessment of liver receptor occupancy after IV administration of the drug may

be a powerful tool to assess differences in intrinsic hepatic clearance among glucocorticoids.

The intratracheal (IT) administration of FP resulted in receptor occupancies which were significantly lower for liver ($AUC_{E\ liver}$=235 %*hr; $p<0.01$, see Table 8.2) and kidney ($AUC_{E\ Kidney}$=481 %*hr; $p<0.04$) than that for lung ($AUC_{E\ Lung}$=637 %*hr). No significant difference was observed between lung and spleen receptor occupancies ($p>0.5$). Pulmonary targeting defined as $AUC_{E\ Lung}$ -$AUC_{E\ liver}$ was also significantly greater than that defined as $AUC_{E\ Lung}$ -$AUC_{E\ Kidney}$ (see Table 8.3). The higher pulmonary targeting observed for lung versus liver data is likely due to the combined effects of high intrinsic clearance, and of pulmonary delivered FP. The highly significant difference between lung and kidney receptor occupancies suggest that the kidney is an adequate organ to assess fluticasone propionate pulmonary selectivity.

The intratracheal administration of TA-dry powder (100 μg/kg), resulted in liver ($AUC_{E\ liver}$=655 %*hr) and kidney $AUC_{E\ Kidney}$=609 %*hr) receptor occupancies which were significantly lower (Table 8.2) than those for lung ($AUC_{E\ Lung}$ =724 %*hr). A comparison between pulmonary targeting defined as $AUC_{E\ Lung}$ -$AUC_{E\ Liver}$ (69 ± 51 %*hr) and $AUC_{E\ Lung}$ -$AUC_{E\ kidney}$ (115 ± 83 %*hr) yielded values no significant difference (see Table 8.3). This results suggested that the higher pulmonary targeting obtained upon IT administration was not affected by the drug intrinsic hepatic clearance, but solely reflects pulmonary targeting. Thus, either liver or kidney are a good makers to assess the extent of systemic absorption of TA upon inhalation.

The intratracheal administration of TA and FP, as well as the intravenous administration of FP resulted in close superimposition of lung and spleen receptor occupancies, indicating that the spleen may not be an adequate systemic organ to assess the degree of glucocorticoid spill over into the systemic circulation. The reason for this pattern is yet to be determined. It is well known that glucocorticoids induce the redistribution of several blood cells to different lymphatic organs including spleen [142]. Thus, one can speculate that this lack of targeting may be due to mobilization of blood cell to this organ.

In the present study, the intratracheal administration of equal doses (100 μg/kg) of FP and TA resulted in no significant difference in pulmonary targeting values when defined as $AUC_{E\ Lung}$ -$AUC_{E\ Kidney}$ ($p>0.05$), although a tendency for higher targeting was indicated for FP. This finding was at first surprising, since the superiority of FP to act as an "airway depot" has been claimed. We have reported earlier that pulmonary targeting is affected by clearance, release rate, and the dose. FP and TA with receptor to binding affinities, differ by a factor of 9 (see Table 2.3, chapter 2), meaning that equal doses of 100 μg/kg will result in quite different doses, for example, expressed as TA equivalents (TA 100 μg/kg versus FP 900 μg/kg). In addition, clinical studies have shown that the absorption profiles of TA and FP are relatively similar, with FP being the slowest dissolved (Hochhaus personal communication). Thus, a lack of difference in pulmonary targeting between TA and FP might be due to either an overdosing of fluticasone propionate, or because indeed, no significant differences in release rate characteristics exist between these two drugs. Future studies are needed to determine the optimal dose of fluticasone propionate that is able to achieve maximum pulmonary targeting.

The intratracheal administration of fluticasone propionate-dry powder resulted in a mean pulmonary effect time (MET=5.5, Table 8.1) which was longer than that after the IT administration of TA-dry powder (MET=4.7). Since the difference is not that pronounced, this finding supports the hypothesis that dissolution rate between these drugs is similar. In contrast, however, mean effect times after IT and IV administration of FP were the same (MET=5.5), however. The reason for these findings are yet to be determined.

The intratracheal administration of triamcinolone acetonide phosphate in 800 nm liposomes (TAP-lip 800 nm; data reproduced from chapter 7) resulted in a significantly higher pulmonary targeting (defined as $AUC_{E\,Lung}$ - $AUC_{E\,Liver}$) and longer mean effect time (MET) than that after triamcinolone acetonide-dry powder administered in the same fashion (Figure 8.4, Table 8.1), indicating the superiority of the liposomal formulation to attain more prolonged pulmonary effects as well as higher pulmonary selectivity.

CHAPTER 9
CONCLUSIONS

The following conclusions were drawn from the series of experiments performed in this research:

1. Triamcinolone acetonide phosphate was successfully encapsulated into liposomes.
2. The liposomal formulation consisting in phosphatidylcholine and phosphadylglycerol (9:1) and triamcinolone acetonide phosphate was stable at 37 °C under sink conditions and in the presence of various fluids including broncolavage fluid.
3. Intratracheal administration of triamcinolone acetonide in liposomes (TAP-lip) provided sustained lung receptor occupancy, and increased pulmonary targeting which were superior to IT administration of the drug in solution or the intravenous administration of the liposomal formulation (TAP-lip). Therefore, pulmonary targeting is not only linked to the slow release characteristics of the drug, but also to the route of administration.
4. The encapsulation of a glucocorticoid into liposomes changed favorably its pharmacokinetics (longer mean residence time and higher local versus systemic drug ratio), as well as its overall pharmacodynamics (longer mean effect time) without changing its intrinsic pharmacodynamics (EC_{50} values and maximum binding), suggesting that pharmacokinetics is the driven force to optimized pulmonary targeting.

5. Physical stability of liposomes (leakage) is determined by the vesicle size: the larger the vesicle the higher the stability against leakage.
6. There exists an inverse relationship between liposome stability and drug release rate from liposomes: the higher the liposome stability the slower the release rate.
7. Pulmonary targeting can be enhanced by slowing down the release rate. It suggests that, drugs with lower dissolution rates may induce higher pulmonary targeting. Since commercially available glucocorticoid have been shown to differ in their dissolution rates, they may also have differences in pulmonary targeting.
8. Mean effect time (average time that lung receptor are occupied) is inversely proportional to release rate: the slower the release rate the longer the mean effect time. Thus, the administration of drug into liposomes may allow once a day administration and therefore, higher patience adherence.
9. There is an optimal dose for which maximum pulmonary targeting can be achieved.
10. Liver may not be an adequate organ to evaluate the extent of systemic absorption of drugs with high intrinsic hepatic clearance such as fluticasone propionate.
11. Apparently, there is not a significant difference in pulmonary selectivity between fluticasone propionate and triamcinolone acetonide when the same dose (100 μg/kg) is administered.
12. Liposomal TAP represents a better approach to optimize sustained delivery of glucocorticoids to the lungs via topical administration, than TA dry-powder formulation.

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BIOGRAPHICAL SKETCH

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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